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A STUDY OF PHYCOPHYSIOLOGY IN CONTROLLED ENVIRONMENTS

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Introduction

This report reviews work under Research Grant No. NsG-70-60 made to the University of Maryland for the period 1 April, 1963, to 31 March, 1965. The initial grant was for a three year period beginning 1 April, 1960, to 31 March, 1963. The period covered by this report includes 1 April, 1964, to 30 September, 1964. The description of the proposed research is given in the proposal for continuation submitted on 15 February, 1963. This report conforms to the requirements from the NASA Office of Grants and Contracts with regard to format and material to be included concerning both financing and reports of research. As in previous reports, details of completed research are found in the manuscripts and reprints provided with the main report.

Research Summary

During future space flights which are expected to be of many weeks duration, the support of man by some type of partial or complete regenerating system for oxygen and food is an inescapable necessity. The logistic effort concerned with the maintenance of men on bases outside the atmosphere of the earth, either on the moon or in orbiting satellite observatories, can be reduced by the degree to which problems of regeneration can be solved. The most useful model for supplying man with his requirements is that which has been most successful and common on earth--essentially a man-plant balanced system-- the plant supplying the needs of man and vice versa. The purpose of the research being conducted at the University of Maryland is to examine in depth one of the most promising components of such a closed ecological system with a view toward its ultimate incorporation into flight systems.

The fundamental problem is to determine how to simplify and compress a functioning plant support-system for man. In any simplification the scientist is forced to exchange the inefficient, but stable and inexpensive natural environment of earth, for a more efficient, but less stable and necessarily more expensive climate of a closed capsule. In such a capsule stability is purchased by the expenditure of large amounts of energy. It will work only if the optimum metabolism of both plant and man are maintained.

Considerable research with many plant species has led to the most careful examination of the unicellular, photosynthetic microorganisms of the genus Chlorella. When illuminated, these algae are capable of the most

efficient conversion of water and carbon dioxide to carbohydrates, fat, and oxygen. When supplied small amounts of nitrogen they synthesize large quantities of protein as well. Consequently, they are proving excellent absorbers of the CO_2 exhaled by man and producers of both the oxygen breathed and the food consumed by man.

In order that a better knowledge of the fundamental physiology and biochemistry of these organisms, which may occupy the closed system with man, may be achieved, research during the past months has centered on four major areas of concern that can be summarized as follows:

1. The study of the growth of Chlorella in a prototype of the life-support unit, called the Recyclostat, has been accelerated. The Recyclostat is a device for the sterile culture of Chlorella grown on a simple nutrient medium which provides for harvesting of the algae, recycling of the medium, and monitoring of production for long periods of time. The best measure of the oxygen production by a culture of algae is the growth rate. Cultures of Chlorella have been grown at satisfactory growth rates for periods exceeding one month without the accumulation of toxic products.

   Such long-term cultures made possible the determination of fluctuations in the rate of growth, the necessary nutrient formula for maintaining growth, and the nature of products secreted by the organism into the medium. Construction and improving of a simple apparatus for the long-term sterile culture of the algae not only has provided a basic tool for studying the biology of closed systems,

but has established the guidelines for the design of flight apparatus for Chlorella or any other similar organism.

2. Because algae may be used for human consumption a careful analytical study of their composition has been instituted. During the last report period, the determination of the chemical structure of the sterols of five species of Chlorella has provided the first sure knowledge that these sterols, so closely related to powerful hormones of the human body, are not of the type likely to be dangerous to man. Poriferasterol, clionasterol, and 22-dihydrobrassicasterol were discovered as sterols never before reported in plants. Interestingly, dihydrobrassicasterol has never been found previously in nature.

3. The effect of light intensity on the shifts in chlorophyll a/b ratios has been established. The total concentration of pigments vital to photosynthesis has been shown to decrease with the increase in light intensity. This has been known, but what is new is that the relative amount of chlorophyll b decreases more with increasing light than does chlorophyll a. This is of importance in the theory of photosynthesis and explains certain other shifts which occur at higher light intensities.

Further studies of the cell growth of Chlorella at high light intensities suggest that the algae secrete buffering compounds into the medium. The major buffering

anion was shown to be bicarbonate, and, under certain conditions, potassium was secreted to increase pH. Changes in the internal cell and the changes brought about by cellular action on the medium are examples of information which is essential if human control of cultures is to result in the optimal production of oxygen and/or food.

4. The metabolic activity of cells of different ages within the cultures has been studied further. All the evidence at hand indicates that in older cells photosynthetic activity is reduced along with metabolism in general. At this stage, oxygen evolution is also reduced. Consequently, this aging in a cell population must be kept at a minimum. This can be done in cultures which are maintained at near their maximal exponential growth rate. Consequently, design and operation of the culture devices must provide for optimal growth rates in order that efficiency can be maintained.

5. Special attention has been devoted to observations on the secretory activity of algal cells. Its effect on the buffering capacity of the medium and on cell division were studied as an extension of findings published previously. The buffering capacity of substances liberated by algal cells was studies in relation to external conditions and was expressed in terms of van Slyke's buffer index calculated as

$$\beta = \frac{dB \text{ (or } dA)}{dpH}$$

where B is the amount of base (A is amount of acid) in milligram equivalents per liter of titrated fluid used to exert a shift in pH equal to 1 unit.

It was confirmed that the secretory activity of cells is a time-dependent phenomenon. In distilled water, the initial pH of 5.7 changed in the course of 4 hours to 7.2 and the buffer index (negligible for distilled water) rose to a value of 0.22. After 20 and 44 hours, the pH rose, respectively, to 8.2 and 8.6 and the buffer index to 0.86 and 1.1. Similar time effects were observed at a lower initial pH.

The effect of the initial pH on the subsequent buffering activity of cells was observed by suspending cells in different concentrations of HCl. In 1×10^{-4} N HCl, the initial pH of 4.4 rose in the course of 44 hours to 8.6 and the buffer index to 0.76. In 1×10^{-3} N HCl, the initial pH increased during the same time from 3.1 to 7.1 and the buffer index to 0.22. In 1×10^{-2} N HCl, the initial pH of 2 did not change during observations. There was no cell secretion.

When different amounts of cell material were suspended in the same volume of 1×10^{-3} N hydrochloric acid, the final pH was found to be much higher at higher population density. At the population densities of 3.49 and 1.75 gm dry weight cells/liter

suspension, the pH rose in the course of 44 hours from the initial level of 3.4, respectively, to 8.7 and 7.7. At the initial population density of 0.85 and 0.35 gm cells/l, the rise in pH was small. The buffering capacity of the suspending fluid was much higher at higher population density not only per unit volume of the suspension, but also in regard to the unit of dry weight of cells. Thus, one gram of dry weight of cells at higher population density secreted more material than one gram of cells did at lower population density. This phenomenon can be possibly understood in terms of two time dependent and inter-dependent processes: one, secretion by the cells of substances of high buffering capacity; another, the damaging effect of low pH on cells, particularly on their secretory activity. The progress in one process undermines the other.

An increase in initial pH above that of distilled water increased cell secretion. Thus, buffering activity of the suspending fluid increased by suspending cells in 2×10^{-3} N and even more in 1×10^{-2} N KOH. Parallel to this, the pH of the suspending fluid declined correspondingly from the initial 11 to 8.7 and from 11.8 to 9.3.

In all observations, though the buffering capacity of the medium was different, depending on external factors and the amount of cell material per volume

of the suspension, the position of the peak on the titration curve was always the same, at about 6.4. This value corresponds to the pK_1 of carbonic acid. The formation of bicarbonate is thus largely responsible for buffering capacity of the medium caused by vital activity of cells. Carbon dioxide for bicarbonate formation is evidently provided by the cells in the process of respiration. In water and in dilute acids as suspending fluids, cations are also secreted by the cells. The major cation was found by flame spectrophotometry to be potassium.

Bicarbonate is not the only component of cell secretions. Work is in progress now indicating that other substances, particularly those with high pK, are often secreted by cells.

The secretory activity of cells depends on several factors, the most important of which, in case of synchronized cells, are:

a. Cells must be physiologically active, vigorously growing on the day previous to experimentation.

b. Cells must be supplied in dark with oxygen.

c. The initial pH of the medium must not be too low.

d. Under given conditions and at a given initial pH, there must be enough cells per volume unit of the suspending fluid (size of the inoculum).

During the current report period, studies in each of the areas in which progress has been reported will continue. If the organisms under study are to serve as components of a support-system for man, no effort must be spared in understanding their physiology and how it reacts as conditions are altered. The availability of such information will provide the necessary data for the construction of long range support systems for the man-in-space program.

Papers

Galloway, Raymond A., Hugh G. Gauch, Jr., and C. John Soeder. The effects of inhibitory levels of CO_2 on Chlorella. Presented at 15th Annual AIBS Meetings at Boulder, Colorado, August 23-28, 1964.

Gross, Rudolph E., and Robert W. Krauss. A physiological examination and taxonomic revision of species of Chlorella isolated from marine habitats. Presented at 15th Annual AIBS Meetings at Boulder, Colorado, August 23-28, 1964.

Karlander, Edward P., and Robert W. Krauss. The mechanism of the light requirement for heterotrophic growth in Chlorella vulgaris Beyerinck. Presented at 15th Annual AIBS Meetings at Boulder, Colorado, August 23-28, 1964.

Patterson, Glenn W., and Robert W. Krauss. Comparative studies of sterols in Chlorella. Presented at 15th Annual AIBS Meetings at Boulder, Colorado, August 23-28, 1964.

Sorokin, C. Ontogenetic aspects of the reaction of photosynthesizing cells to light. Presented at the Fourth International Photobiology Congress, Oxford, England, July 26-30, 1964.

Sorokin, C. Carbon dioxide, pH, and buffering activity of cells in cell division. Invitational paper presented at the Symposium on Cell Division and Cell Differentiation, held at the Tenth International Botanical Congress, Edinburgh, Scotland, August 3-12, 1964.

Sorokin, C. Introduction and historical review at the Symposium on the Developmental Aspects of Cell Metabolism (of which I was an organizer and chairman) held at the 15th Annual AIBS Meetings at Boulder, Colorado, August 23-28, 1964.

Publications

Sorokin, C. 1964. Aging at the cellular level. Experientia 20: 353-362.

Sorokin, C. 1964. Organic synthesis in algal cells separated into age groups by fractional centrifugation. Archiv für Mikrobiologie 49: 193-208.

Plans for the Future

The coming report period should be devoted to a continuation of the current effort. It will be especially concerned with the re-evaluation of data collected in the recyclostat thus far. Data will be assembled and evaluated with special regard to the degree of rhythm which one can expect during the course of continuance of growth. A continuation of the study of the special effects of light is projected with a view toward the completion of an action spectrum for the inhibition of growth of colorless organisms by light on the visible spectrum.

Much data on many physiological phenomena of algae is now at hand and the coming period should see much of this reduced to a form suitable for publication.

Photosynthesis in Cell Development*

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Summary

Nonsynchronized suspensions of the green, high-temperature alga Chlorella 7-11-05 were subjected to fractional centrifugation, and two size groups of cells were separated. The small-cell fraction was presumed to consist largely of younger cells and the large-cell fraction predominantly of older cells. Manometric measurements in phosphate buffer at pH 4.5, in bicarbonate buffer at neutral pH, and in carbonate-bicarbonate buffer at pH 9.3 indicated that younger cells invariable possessed higher photosynthetic activity than older cells, provided the separation of cells into size fractions was reasonable good and the large cells were prevented from dividing during the process of separation. The superior activity of younger cells was ascertained at various light intensities and at different temperatures.

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Previous observations on the decline in photosynthetic activity with the age of synchronized cells were thus substantiated in the absence of a synchronizing agent, and the decline in photosynthetic capacity must be assumed to be characteristic of normal cell development. The decline in metabolic activity in the course of cell development is discussed in connection with metabolic turnover and is viewed as a demonstration of aging of cells.

Introduction

Photosynthetic activity in synchronized algal cells has been shown to decline with the progress in cell development (1, 2). Older cells have lower rates of photosynthetic gas exchange in both light-limited and light-saturated sections of the light intensity curve (2). Photosynthesis in older cells is saturated at lower light intensity (2, 3). Older cells are more sensitive to the deleterious effects of strong light (2). Relative quantum efficiency is lower in cells approaching the end of the life cycle (4). The inferior photosynthetic capacity of older cells was observed under various experimental conditions: in a wide range of light intensities (5), at different temperatures (6), and in several suspending fluids (7, 8, 9).

Yet the significance of extensive studies on synchronized cells depends upon the assumption that observations on synchronized cells reflect upon metabolic events inherent to the normal life cycle of cells. The effect of synchronization technique, as of any other environmental condition, cannot be easily dismissed. The extent of distortion of the course of metabolic activity inflicted by a synchronizing agent has been, however, uncertain. The core of the problem lies with the question whether this distortion is only of a degree or of such proportion that observations on synchronized cells

are not descriptive of metabolic events characteristic of the developmental cycle of normal cells in the absence of the effects of a synchronizing agent.

The technique chosen to investigate the problem consisted in comparative measurements of photosynthetic activity in cells separated into age groups from a nonsynchronized population by fractional centrifugation. The small-cell fraction was expected to consist mostly of younger cells and the large-cell fraction predominantly of older cells. Before being separated, cells of these two fractions had been subjected to the same environmental conditions. The difference, if any, in their performance after separation could be safely ascribed, therefore, only to the difference in the age composition of these cell fractions.

Material and Methods

Two considerations affected the choice of a technique for growing cells for contemplated observations. First, nonsynchronized cell suspensions to be subjected to centrifugation must have the widest possible dispersion of cell sizes to permit sharper separation of small cells from large cells. Second, the amount of cell material subjected to centrifugation must be large enough so that, even after repeated centrifugations and elimination of the major portion of intermediate cells, enough material is left for photosynthetic measurements as well as for cell volume and dry weight determinations.

To satisfy the above stipulations, a nonsynchronized stock suspension of the green, high-temperature alga Chlorella 7-11-05 (10) was maintained as a batch culture under conditions permitting optimal growth. The experimental setup, nutrient medium, and carbon dioxide supply were as previously described (11). Temperature was maintained at 38°, and light intensity from cool-white fluorescent lamps at about 2,000 foot candles. On the eve of the experiment

(4:00), 800 ml of nutrient medium were inoculated with the stock culture, poured in a number of culture vessels (11), and placed back into the same growth bath.

During 16 hours of growth (the experiments were started the next morning at 8:00), the optical density increased about 100 fold, from 0.03 to about or above 3. The 100-fold increase indicated that from 6 to 7 doublings of cell materials occurred during this period. The growth rate, calculated for 24-hour period, was close to 10, indicating that optimal conditions were maintained throughout growth period (12). The final optical density of the suspension corresponded to about 2 gm. dry weight of cells per liter of the suspension.

Microscopic examination of cells at the time of harvesting revealed a wide dispersion of cell sizes: from 1 to 2 μ in diameter for daughter cells to 11 or 12 μ for well-developed mother cells. The latter were close to cell division or were dividing into as many as 32, 64, and probably even more daughter cells.

After being harvested, cells were centrifuged for 10 minutes at 1,000 g and resuspended in 0.02M KH_2PO_4 buffer at pH 4.5. The low pH of the suspending fluid during centrifugations of cells prevented large cells from dividing (13, 14) and thus guaranteed constancy of size composition of the large-cell fraction throughout centrifugation procedure.

After resuspension in phosphate buffer, cells were centrifuged for 5 minutes at 200 g (International centrifuge, conical bottom centrifuge tubes, 50-ml capacity) and the supernatant containing the small-cell fraction was separated from the deposit. The deposit containing the large-cell fraction was again resuspended in the same phosphate buffer. The small- and large-cell fractions were then subjected to repeated centrifugations (three for each kind

of cell) and after each centrifugation the deposit from the small-cell fraction and the supernatant from the large-cell fraction were discarded.

The intensity of centrifugation was determined as a compromise between the objective to have enough cells to carry out observations and the attempt to obtain the best possible separation of small and large cells. The duration of repeated centrifugations usually varied from 2 to 5 minutes, and the speed from 100 to 200 g. In practice, the decision as to the duration and speed of each centrifugation for each fraction of cells was made on the basis of previous experience and continuous microscopic observation of cells in both fractions obtained after each centrifugation.

Attention is drawn to the fact that by this technique, a considerable portion of cells of intermediate size (and age) was discarded from both the small- and the large-cell fractions. The severity of separation of intermediate cells can be judged by considering that after all centrifugations were completed, both the small- and the large-cell fractions contained together only from about 1/20 to 1/10 of the cell mass (on dry weight or cell volume basis) originally present in the suspension at the time of harvesting.

After the last centrifugation was completed, the large-cell fraction was resuspended in the desired suspending fluid, and both the small- and large-cell fractions were brought, by dilution, to the same optical density with the objective of having approximately the same amount of cell mass in a volume of suspension. If measurements were made in a suspending fluid other than phosphate buffer, then the small-cell fraction was centrifuged for 10 minutes at 1,000 g and the cells resuspended in the desired fluid.

Measurements of photosynthetic gas exchange were made with manometric technique in rectangular flasks by either the one-vessel or two-vessel method (15). If cells were suspended in phosphate or bicarbonate buffers,

manometric flasks, after pipetting algal suspensions, were gassed with 5 per cent carbon dioxide. Illumination was provided by banks of incandescent lamps placed beneath the glass bottom of the bath. Light intensity measurements were made with Weston illumination meter (Model 756) with the photocell held in the bath at the level of manometric flasks.

Calculations were made by standard technique applied to measurements by one-vessel or two-vessel method (15). No correction for respiration was made. To eliminate inaccuracy of data based on an assumed assimilatory quotient, data obtained in phosphate and bicarbonate buffers with one-vessel method were also calculated by a method in which manometric readings were corrected only for differences in the gas volume in different flasks and for differences in the amount of cells per flask in different experiments (7). The relative data obtained by this method of calculation are plotted against time in Fig. 2-6.

Packed cell volume was determined by centrifugation of aliquots of cell suspension in calibrated capillary tubes. Determinations of dry weight of cells were made by drying aliquots of cell suspensions to a constant weight at 100°.

Results

The degree of separation of cells into two fractions as dependent on the number of centrifugations was determined in one of the experiments and is shown as polygons of distribution of cell diameters in two fractions of cells (Fig. 1). After two centrifugations, the median for the small-cell fraction was at 2.4-3.6u and for the large-cell fraction at 3.6-4.8u. Separation improved after three and even more after four centrifugations.

The median for the small-cell fraction was after four centrifugations at 1.2-2.4μ and for the large-cell fraction at 6-7.2μ.

None of the cells in the small-cell fraction exceeded 3.6μ in diameter, and 91 per cent of cells in the large-cell fraction were above this size. However, even after four centrifugations, separation of cells into two fractions was not perfect: 9 per cent of the cells in the large-cell fraction were less than 3.6μ in diameter, that is, of the same size as some cells in the small-cell fraction. The experience showed that standardization of all factors involved in the separation of cells by centrifugation is difficult to achieve, and the degree of separation differs from one experiment to another. For that reason, observations are readily comparable only within one experiment.

Time course of gas exchange in two fractions of cells separated after four centrifugations is shown in Fig. 2-7. Measurements made in phosphate and bicarbonate buffers are calculated in relative units (Fig. 2-6); those made in Warburg buffer No. 9 (Fig. 7) in terms of evolved oxygen. In phosphate buffer at pH 4.5, the small-cell fraction exhibited a higher rate of photosynthetic gas exchange in a wide range of light intensities (Fig. 2-4). The intensity of 4,000 foot candles (Fig. 2) is well above light saturation as determined for nonsynchronized suspensions of this strain. The intensities of 700 (Fig. 3) and 1,400 (Fig. 4) foot candles are within the light-limited portion of the light intensity curve. A comparison of the time course curves obtained at 39° (Fig. 4) with those at 30° (Fig. 5) indicates that the superior activity of the small-cell fraction can be seen at both temperatures.

A higher photosynthetic capacity of the small-cell fraction was also demonstrated in bicarbonate buffer at neutral pH (Fig. 6) and in terms of evolved oxygen in Warburg buffer No. 9 at pH 9.3 (Fig. 7).

Photosynthetic activity of the small- and large-cell fractions in terms of oxygen evolved during the first hour of observations is reported in Table 1. Measurements in phosphate buffer were done by the two-vessel method; in bicarbonate and Warburg No. 9 buffers by the one-vessel method. Calculations of data obtained for bicarbonate buffer are based on an assumed assimilatory coefficient ($CO_2/O_2 = 0.9$).

Photosynthetic rates were generally lower than those previously reported for nonsynchronized suspensions of Chlorella 7-11-05 (10, 16). These low rates were probably due to the elimination in the process of centrifugation of the large portion of cells of intermediate sizes (and ages) previously found to have the highest photosynthetic activity (1, 2). In different experiments different amounts of intermediate cells were removed. For this reason, photosynthetic activity of the small- and large-cell fractions is comparable only within one experiment.

Rates of oxygen evolution were generally higher for the small-cell fraction. However, the difference in photosynthetic activity between these two fractions of cells varied from one experiment to another, probably due to differences in the degree of separation of these two fractions of cells in various experiments. In Warburg buffer No. 9, there was no difference in the performance of the small- and large-cell fractions during the first hour of observation.

As previously indicated, older cells differ from younger cells not so much in the level of photosynthetic activity as measured for short time intervals at the beginning of observation as in the pace with which the activity changes with time in prolonged experiments (5,7). The level of photosynthetic activity for three consecutive hours of one experiment is shown for the large-cell fraction in per cent of that for the small-cell fraction.

in Table 2. Since the dry weight of cells per unit of packed volume of cells may change with the age of cells, calculations are made on both cell volume and dry weight basis. In the majority of the experiments, the difference in the performance of the large-cell fraction relative to that of the small-cell fraction, on both cell volume and dry weight basis, greatly increased with time.

Discussion

The superior photosynthetic activity of younger cells separated from older cells by fractional centrifugation has been demonstrated in these experiments under various external conditions. The decline in photosynthetic activity with the age of cells, previously reported for synchronized Chlorella cells suspended in phosphate buffer (1, 3, 7), bicarbonate buffer (8), and Warburg buffer No. 9 (9), was thus confirmed in all these buffers on cells mechanically separated into age groups from nonsynchronized populations of cells. The effect of the age of cells on their activity was demonstrated at different light intensities and at different temperatures.

The concern has been expressed that, due to the effects of the synchronizing agent, observations on synchronized cells may not be characteristic of nonsynchronized cells passing through their normal developmental cycle (17-20). These doubts received support from contradictory observations made on cells separated into age groups by fractional centrifugation by the late Dr. R. Emerson. In one report Emerson indicated that the large - i.e., older - cell fraction has a higher quantum requirement per molecule of photosynthetically produced oxygen (21). Later he commented that the difference in quantum efficiency of large and small cells was transient and, therefore, not real (22). Since no

description of the technique was published, it can be surmised that the opposite pronouncements were caused by differences in the technique used in the two sets of observations, possible in the technique of separation of cells into age groups.

Separation of cells into age groups by fractional centrifugation is based on understanding that large cells, settling during centrifugation to the bottom of a centrifuge tube, are old cells and that small cells left in the supernatant are young cells. This condition is best materialized in a healthy, vigorously growing population consisting of cells with a wide range of sizes and, therefore, of developmental stages. A slow growth under suboptimal conditions may produce cells rather old as to their time (calendar) age but still small in size. Separation of cells into size groups from such a suspension by fractional centrifugation is uncertain, and observations on separated cells are unconvincing. Vigorous growth under optimal conditions is the first condition in studies on cells separated by centrifugation into age groups.

Separation by centrifugation into age groups is facilitated if the difference in size between young and old cells is large; if, therefore, a cell grows to a volume several times larger than that of the cell at the beginning of its development and then divides into a large number of daughter cells. This condition prevails in the high-temperature strain, Chlorella 7-11-05, which, under optimal growth conditions, produces from one mother cell up to 32-64 and even more daughter cells. The diversity in size of the population of cells subjected to centrifugation is the second condition for the successful application of this technique.

Since the resolution of centrifugation technique is rather poor,it is advisable to repeat centrifugation and to discard cells of intermediate sizes (of intermediate developmental stages). It has been shown that photosynthetic

activity during the initial part of the life cycle may rise and reach a peak in cells of intermediate age (size) (1). Only after this, the activity declines to a low level during the second part of the life cycle extended up to or close to the moment of cell division. Depending on subtle variations in centrifugation technique, the majority of cells of intermediate sizes may go into the small- or into the large-cell fraction or may be distributed between these two fractions more or less evenly. To avoid the hazards of such unpredictable distribution of cells of intermediate sizes, the small- and large-cell fractions, after their initial separation from each other, are repeatedly centrifuged at a proper speed and, after each centrifugation, the supernatant is discarded from the large-cell fraction and the deposit is discarded from the small-cell fraction. Repeated centrifugations and elimination of the major portion of cells of intermediate sizes are the third and fourth conditions for using the centrifugation technique.

Precautions must be taken to prevent large (old) cells from dividing in the process of their separation by centrifugation. Failure to fulfill this condition results in transformation of the large-cell fraction into a mixture of large and small cells in which small cells may become predominant with time. Inhibition of cell division may be achieved by working at lower temperature, by using inhibitors, or by suspending cells in a medium with low pH. In our work, suspension of cells in phosphate buffer at pH 4.5 was adequate for this purpose. Arresting cell division during separation of cells is the fifth condition essential for the technique.

Finally, measurements of photosynthetic activity in the small- and large-cell fractions may produce inconclusive results due to the fact that, depending on their exact developmental stage, young cells may have the same or even lower activity as older cells if measured for a short period of time.

However, young and old cells differ in the way the activity changes with time. In young cells at a certain developmental stage and under favorable conditions, the activity increases with time. Under less favorable conditions it decreases with time. However, under the same conditions, the rate of the increase in younger cells is higher and the rate of the decline is smaller than that in older cells. Thus, most characteristic of the young and old cells is the direction and the rate of change in their activity measured under the same experimental condition. Prolonged observations on the change in the activity of the small- and large-cell fractions is the sixth condition for the unambiguous evaluation of the relative photosynthetic capacity of young and old cells.

Changes in photosynthetic activity in the course of cell development must be accepted as a biochemical reality bound with aging of cells. Deterioration of photosynthetic mechanisms responsible for the decline in photosynthetic activity is evidently caused by the increase in catabolic activity and by the decline in anabolic activity with the age of cells. Both moieties of the photosynthetic machinery - one connected with the dark enzymatic reactions and another responsible for the primary photochemical act - are probably involved. The involvement of both parts of the photosynthetic apparatus is indicated by lower photosynthetic capacity of older cells at both light-limiting and light-saturating conditions. Actually, metabolic turnover has been generally demonstrated for both enzyme systems (23) and photosynthetic pigments (24-26).

To the effects of environmental factors and physiological condition of cells affecting photosynthetic activity has been added a new parameter -- aging of cells. Studies on quantum efficiency (4), on enhancement effect (27, 28, cf. also 29), and on fluorescence of photosynthetic pigments (30)

strongly suggest that variations and often-recorded inconsistency of observations actually depend on change in age composition of experimental material.

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Legends

Fig. 1 Distribution of polygons of cell diameter in the fractions of small and large cells of Chlorella 7-11-05 as affected by number of centrifugations.

Fig. 2 Time course of gas exchange in small and large cells of Chlorella 7-11-05 separated from a nonsynchronized suspension by fractional centrifugation. Measurements in 0.02M KH_2PO_4 pH 4.5, at 39°, and light intensity of 4,000 foot candles.

Fig. 3 Time course of gas exchange in small and large cells of Chlorella 7-11-05 separated from a nonsynchronized suspension by fractional centrifugation. Measurements in 0.02M KH_2PO_4 pH 4.5, at 39°, and light intensity of 700 foot candles.

Fig. 4 Time course of gas exchange in small and large cells of Chlorella 7-11-05 separated from a nonsynchronized suspension by fractional centrifugation. Measurements in 0.02M KH_2PO_4 pH 4.5, at 39°, and light intensity of 1,400 foot candles.

Fig. 5 Time course of gas exchange in small and large cells of Chlorella 7-11-05 separated from a nonsynchronized suspension by fractional centrifugation. Measurements in 0.02M KH_2PO_4 pH 4.5, at 30°, and light intensity of 1,400 foot candles.

Fig. 6 Time course of gas exchange in small and large cells of Chlorella 7-11-05 separated from a nonsynchronized suspension by fractional centrifugation. Measurements in 0.004M bicarbonate buffer pH 6.9, at 39°, and light intensity of 2,100 foot candles.

Fig. 7 Time course of gas exchange in small and large cells of Chlorella 7-11-05 separated from a nonsynchronized suspension by fractional centrifugation. Measurements in 0.1M carbonate-bicarbonate (Warburg #9) buffer pH 9.3, at 39°, and light intensity of 2,100 foot candles.

Table 1. Photosynthetic activity in small and large cells of Chlorella 7-11-05 separated from a nonsynchronized suspension by fractional centrifugation. Oxygen evolution *during* the 1st hour of observations in $mm^3 O_2/mm^3$ packed cells/hour

Suspending fluid	pH	Conditions during measurements t^o	l.i. (f.c.)	Small cell fraction Individual measurements I	II	III	Average	Large cell fraction Individual measurements I	II	III	Average	Remarks
KH_2PO_4	4.5	39	4000	65	78		72	42	47		45	Two-vessel method
KH_2PO_4	4.5	39	2100	91	107		99	89	93	86	89	" "
KH_2PO_4	4.5	39	1400	118	106	115	113	90	97		94	" "
KH_2PO_4	4.5	39	700	73	76	66	72	45	43		44	" "
KH_2PO_4	4.5	30	1400	88	84	82	85	74	76		75	" "
Bicarbonate	6.8	39	2100	104	105		105	88	96		92	One-vessel method Calculations based on assumed $\frac{CO_2}{O_2} = 0.9$
Warburg #9	9.3	39	2100	101	106	105	104	105	104		105	One-vessel method

Table 2. Change in photosynthetic activity in small and large cells of Chlorella 7-11-05 in the course of one experiment. Rates of photosynthetic gas exchange for the large-cell fraction during each of the three consecutive one-hour intervals in per cent of the corresponding rates for the small-cell fraction.

Suspending fluid and pH	Light intensity, f.c.	Basis for calculations	Hourly intervals I	II	III
KH_2PO_4, pH 4.5	4000	cell volume	64.5	71.0	72.0
		dry weight	63.6	69.9	70.9
KH_2PO_4, pH 4.5	2100	cell volume	89.1	57.0	31.9
		dry weight	90.2	57.7	32.3
KH_2PO_4, pH 4.5	1400	cell volume	100.2	83.3	83.9
		dry weight	103.9	86.2	87.1
KH_2PO_4, pH 4.5	700	cell volume	79.3	61.3	-
		dry weight	81.4	62.9	-
Bicarbonate, pH 6.8	2100	cell volume	88.6	72.6	60.7
		dry weight	93.2	76.4	63.9
Warburg No. 9, pH 9.3	2100	cell volume	100.4	82.0	65.7
		dry weight	105.6	86.3	69.2

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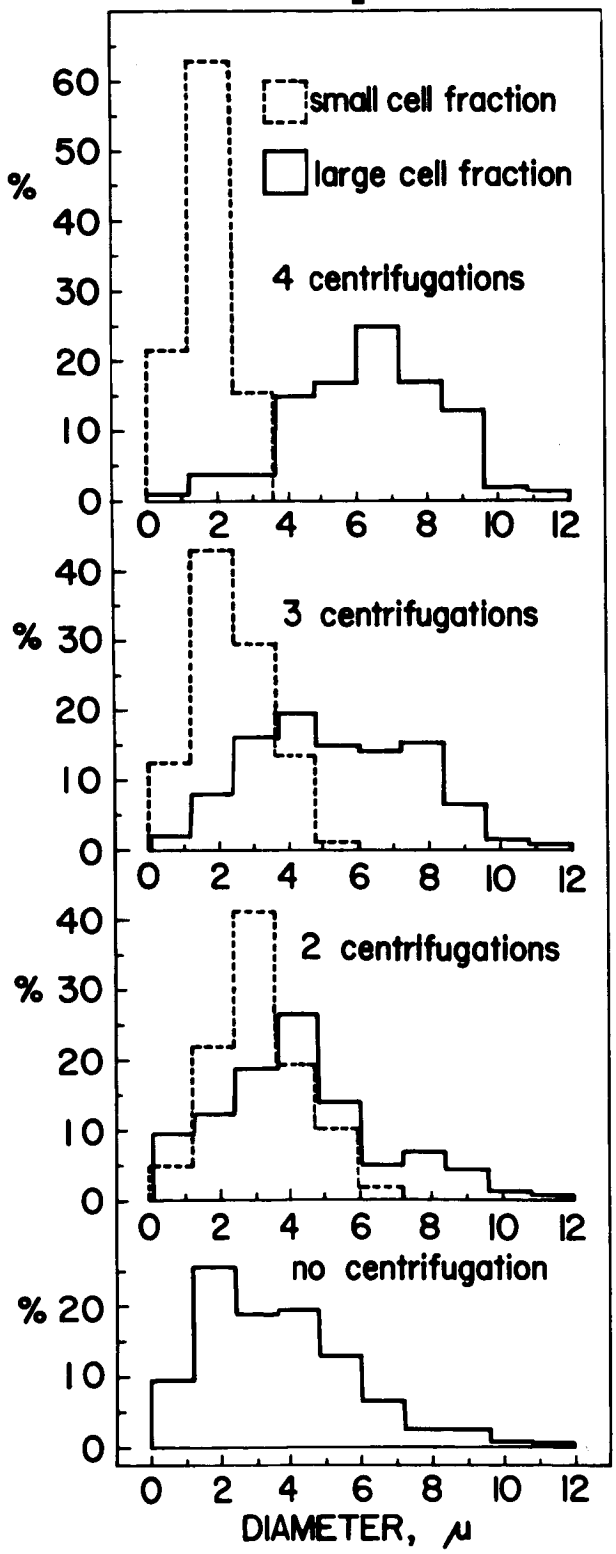
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3 centrifugations
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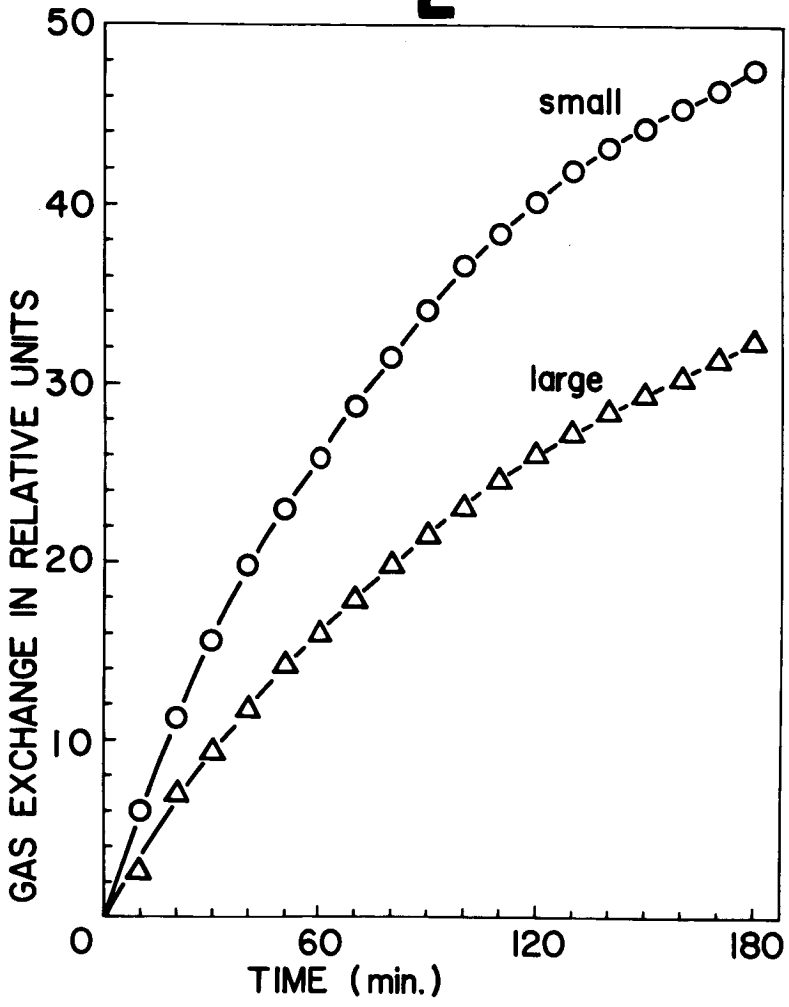
DIAMETER, μ

2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large
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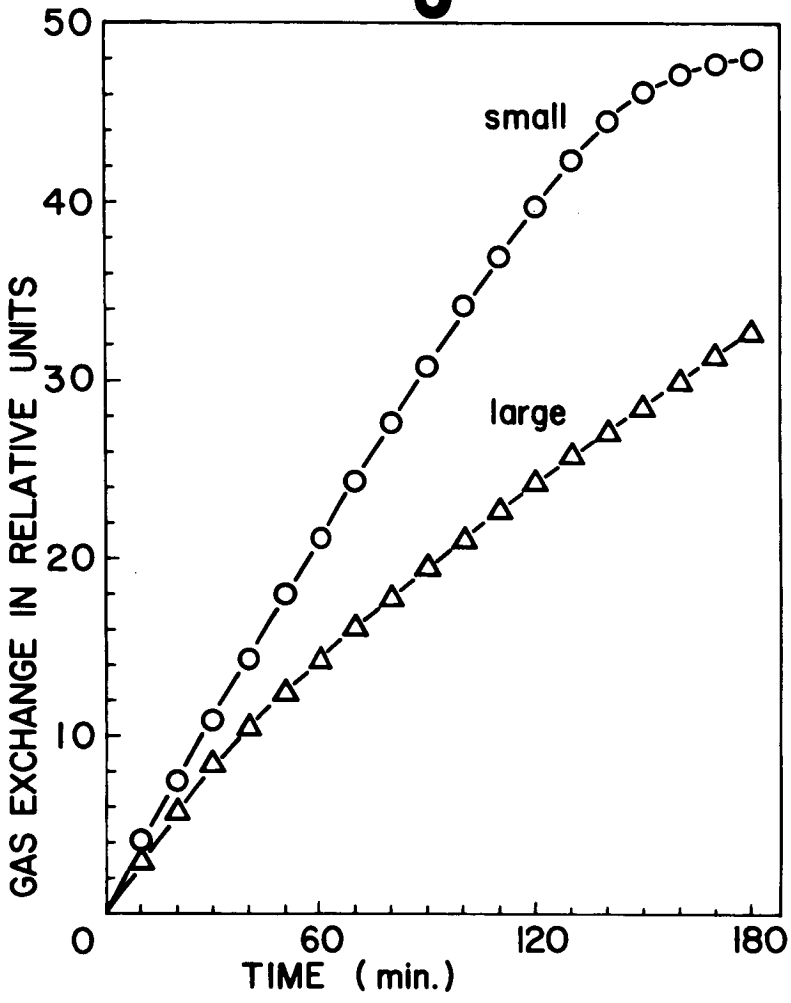
180
TIME (min.)

3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TIME (min.)
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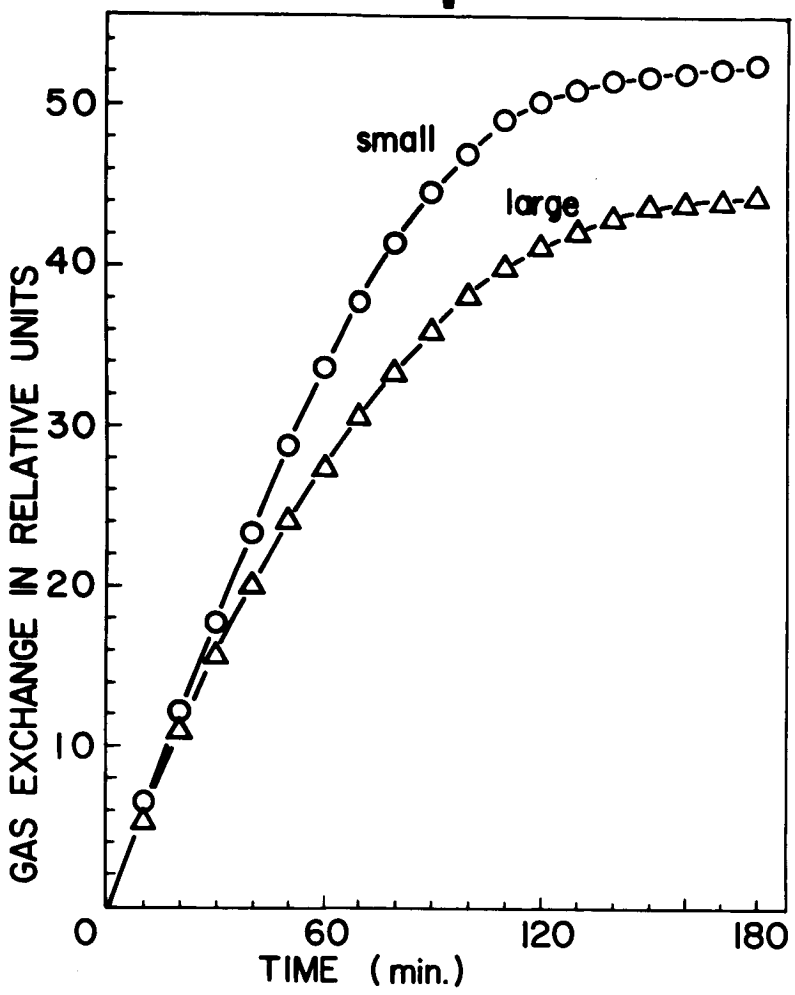
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TIME (min.)
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large

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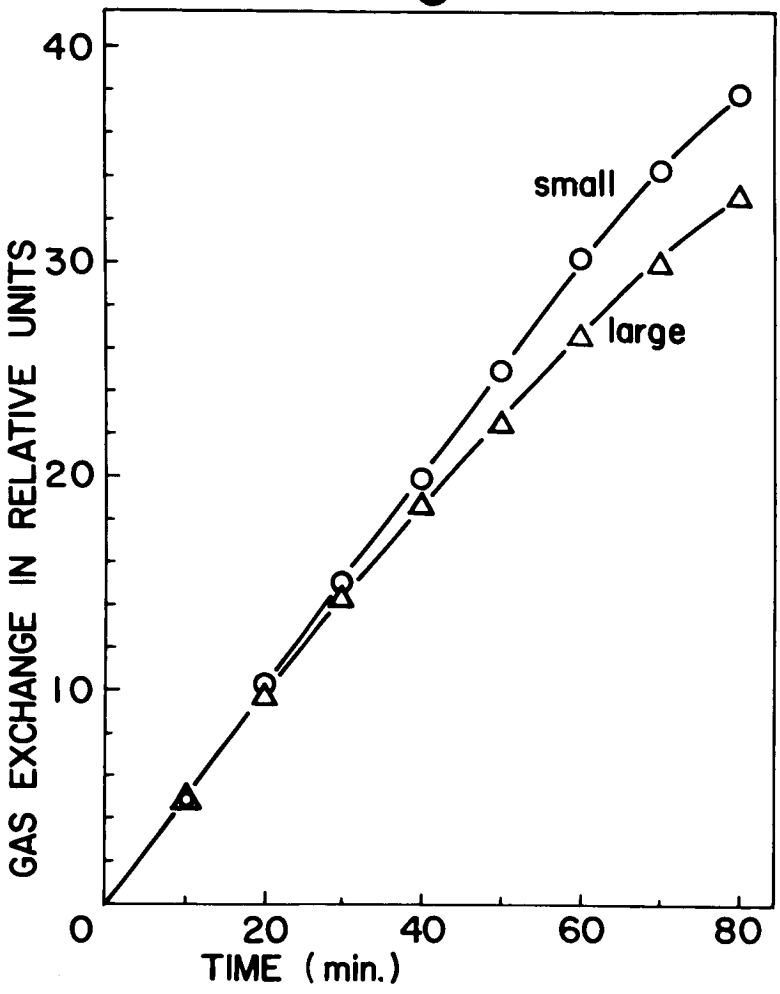
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TIME (min.)
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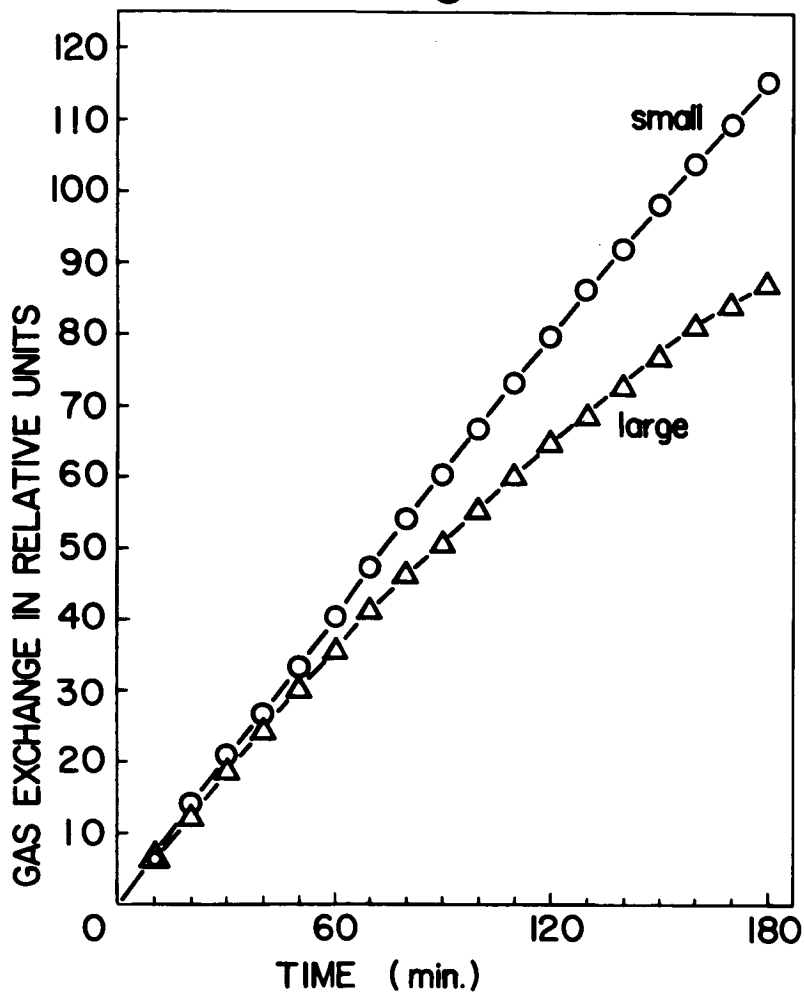
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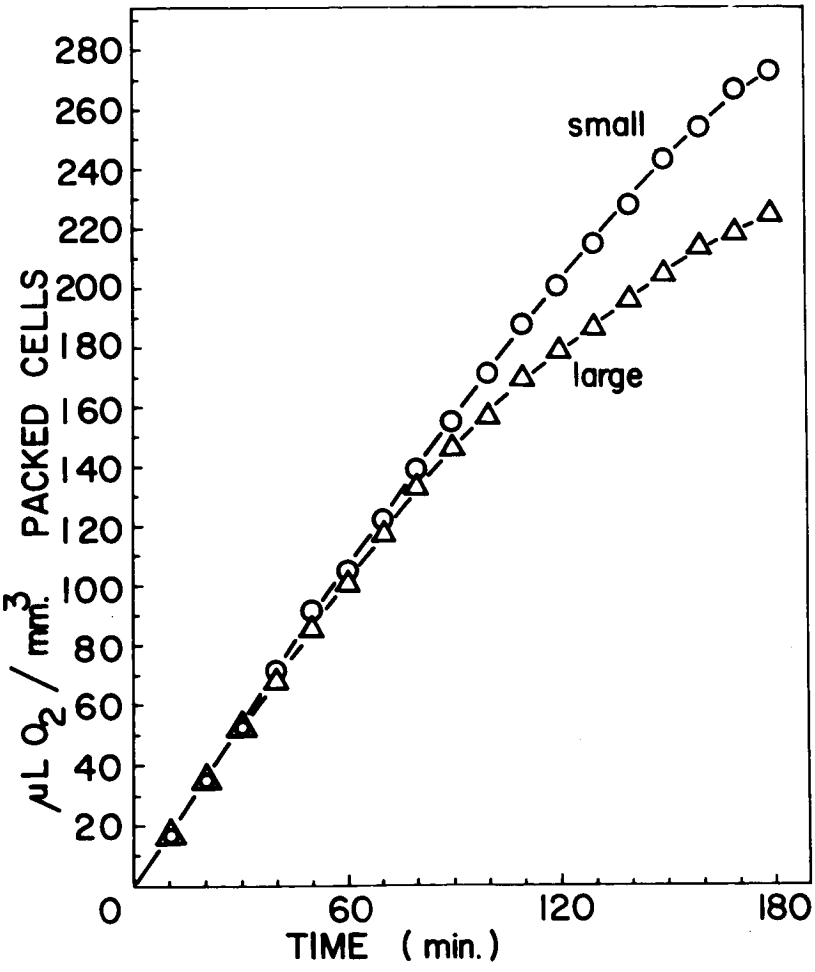
TIME (min.)
small
large

7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μL O2 / mm3 PACKED CELLS
small
large
60
120
180
TIME (min.)

Van Slyke's Buffer Values for Cell Secretions[1, 2]

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(Received)

The capacity of cells to affect pH of the surrounding medium has been ascribed to the amassment in the medium of substances of high buffering capacity (1). Accumulation of these substances is due to the vital activity of cells. The rate of accumulation and the final amount amassed depend on a number of factors of which physiological condition of cells, external factors during and prior to observation, and population density of cell suspension are of major importance. In subsequent investigations, an attempt was made to characterize cell secretions in terms of van Slyke's buffer index (2) and to identify the major component of the buffer system. The term "cell secretions" is used here without implying the mechanism of release or the physiological role of the process. It simply indicates substances liberated by cells into the surrounding medium.

Material and Methods

Observations were made on synchronized suspensions of the green, high-temperature alga Chlorella 7-11-05 (3). Cells were grown under

optimal conditions in a medium described previously (4, 5). Synchronization of cells was achieved by intermittent light:dark technique (6, 7), and the 7-hour-old cells were used for the experiments. Synchronized cells were employed to avoid complexity of the change in the age composition in nonsynchronized populations of cells and to have an opportunity for comparison wity previous observations made on synchronized algal suspensions (1, 4, 8, 9).

After growing in light for 7 hours, synchronized cells were centrifuged, washed in distilled water, and resuspended in a known volume of one of the suspending fluids. The pH of cell suspensions was measured with Beckman Zeromatic pH meter. The amount of cells used in each experiment was determined in terms of their dry weight per volume unit of the suspension. Cell suspensions were placed in the dark at 35° and supplied with atmospheric air passed through ascarite and water. After a period of time, cells were centrifuged out of the medium, and supernatant was used for obtaining a titration curve. Titration was done by measuring amounts of HCl or KOH of known normality necessary to exert a certain shift in pH. Buffer indexes (β) were then calculated according to van Slyke's equation (2):

$$\beta = \frac{dB \text{ (or } dA)}{dpH}$$

where B is the amount of base (A is amount of acid) in gram equivalents per liter of titrated fluid used to exert a measured shift in pH.

In the above differential ratio, dpH is an infinitesimal change in pH, and dB and dA are infinitesimal parts of an equivalent of base or acid. Buffer values based on determinations over measurable increments in pH are only approximately correct, the degree of approximation being

inversely related to the size of the pH interval used during titrations (10).

In actual titrations, buffering capacity was measured over 0.3 to 0.5 unit of pH. In the following graphs, it is expressed in milligram equivalents of acid or alkali necessary to change pH by one unit in one liter of the titrated fluid. Values of β have been also referred to 0.2 unit of pH (11).

In those experiments in which different amounts of cell material were used per volume of the suspending fluid, buffering capacity of the fluid is referred to the dry weight of cells used. Thus,

$$\beta_{D:W.} = \frac{\beta}{\text{gm dry weight cells/l suspension}}$$

Results and Discussion

As the result of cell activity, the pH and buffering capacity of the media changed (Table 1 and Figs. 1-4). In distilled water the initial pH of cell suspension was 5.7 (Table 1). The buffering capacity of water was negligible (Fig. 1). In the course of 4 hours, the pH increased to 7.2 and the maximum buffering capacity rose to a value of 0.22. After 20 hours, the pH reached 8.2 and the buffer index 0.86. After 44 hours, the pH stabilized at 8.5-8.7 and buffer index at $\curvearrowright$ 1.1.

Similar time effect on the increase in pH of the suspending fluid was observed at a lower initial pH. In 1×10^{-4} N HCl, the initial pH of 4.2 increased after 4 hours to 6.7, after 20 hours to 7.7, and after 44 hours it stabilized at 8.5-8.6 (Table 1).

The effect of the initial pH on the subsequent buffering activity of cells and on the change in pH of the suspending fluid is shown in Fig. 2

and Table 1. In 1×10^{-4} N HCl, the initial pH of 4.4 rose in the course of 44 hours to 8.6 and the buffer index to 0.76. For comparison, data for cell suspension kept for 44 hours in H_2O (from Fig. 1) are plotted also in Fig. 2. In 1×10^{-3} N HCl, the pH increased during the same time from 3.1 to 7.1 and the buffer index to 0.22. In 1×10^{-2} N HCl, the initial pH of 2 did not change during observation. There was no cell secretion and no buffering capacity in the medium.

The effect of population density on the secretory activity of cells is shown in Table 1 and Figs. 3 and 4. Different amounts of cell material were suspended in the same volume of 1×10^{-3} N hydrochloric acid. At population density of 3.49 gm dry weight cells/liter suspension, the pH rose in the course of 44 hours from the initial level of 3.4 to 8.7. At population density of 1.75 gm cells/l, the pH rose to 7.7. At 0.85 and 0.35 gm cells/l, the rise in pH was small. The buffering capacity of the suspending fluid was much higher at higher population density not only per unit volume of suspension (Fig. 3), but also in regard to the unit of dry weight of cells (Fig. 4). Thus, one gram of dry weight of cells at higher population density secreted more than one gram of cells did at lower population density. This phenomenon can be understood in terms of two time dependent and interdependent processes: one, secretion by cells of substances of high buffering capacity; another, the damaging effect of low pH on cells, particularly on their secretory activity. The progress in one process undermines the other.

As seen in Fig. 2, the increase in buffering activity of cells was favorably affected by the increase in the initial pH of the suspending fluid. Further increase in initial pH achieved by addition of KOH brought further increase in buffering activity. The addition of 2×10^{-4} N

KOH had no effect on buffering capacity of the medium compared with distilled water (Fig. 5). The buffer index of the suspending fluid increased in 2×10^{-3} N and even more in 1×10^{-2} N KOH (Fig. 5). Parallel to this, the pH of the suspending fluid declined in the course of 44 hours correspondingly from the initial 11 to 8.7 and from 11.8 to 9.3 (Table 1). In 5×10^{-2} N KOH, the change in pH was small, and the buffer index after 44 hours was lower than in 1×10^{-2} N (Table 1 and Fig. 5).

In all the above figures (1-5), though the buffering capacity of the medium was different depending on external factors and the amount of cells per volume of the suspension, the position of the peak was always the same, at about 6.4. This value corresponds to pK_1 of carbonic acid. Indeed, titration curves for blank solution of 1×10^{-3} M bicarbonate and for supernatant from one of our experiments correspond rather well in their shape and in the position of their maxima (Fig. 6). The formation of bicarbonate is thus largely responsible for buffering capacity of the medium caused by vital activity of cells. The nature of the buffer as bicarbonate was confirmed also by manometric determination of CO_2 expelled from bicarbonate by the addition of sulfuric acid. Carbon dioxide for bicarbonate formation is evidently provided by the cells in the process of respiration. In water and in dilute acids as suspending fluids, cations are also secreted by the cells. The major cation was proved by flame spectrophotometry to be potassium.

Summary

Van Slyke's buffer indexes (β) were determined for secretions of synchronized cells of the green, high-temperature alga, Chlorella 7-11-05. The change in pH and the value of the buffer index were found to depend

on initial pH, duration of observation, and population density. The major component of the secreted buffer system was identified as bicarbonate, and the major cation, as potassium.

author

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Table 1. Changes in pH in cell suspensions of Chlorella 7-11-05 kept in dark at 35° in free from carbon dioxide air.

Suspending fluid	Concentration, gm. equival/liter	Population density, gm dr. weight cells / l. suspension	Duration of experiment, hrs.	pH of cell suspension	
				Before	After
(1)	(2)	(3)	(4)	(5)	(6)
H_2O	-	1.4	0	5.7	
	-	"	4	"	7.2
	-	"	20	"	8.2
	-	"	44	"	8.5
	-	"	68	"	8.6
	-	"	92	"	8.7
HCl	1×10^{-4}	1.73	0	4.2	
	"	"	4	"	6.7
	"	"	20	"	7.7
	"	"	44	"	8.6
	"	"	68	"	8.5
HCl	1×10^{-4}	1.15	44	4.4	8.6
	1×10^{-3}	"	"	3.1	7.1
	1×10^{-2}	"	"	2.0	2.0
HCl	1×10^{-3}	3.49	44	3.4	8.7
	"	1.75	"	3.3	7.7
	"	0.85	"	3.2	3.8
	"	0.35	"	3.0	3.3

Table 1. Changes in pH in cell suspensions of Chlorella 7-11-05 kept in dark at 35° in free from carbon dioxide air (cont'd)

(1)	(2)	(3)	(4)	(5)	(6)
KOH	2×10^{-4}	2,72	44	6.6	8.4
	2×10^{-3}	"	"	11.0	8.7
	1×10^{-2}	"	"	11.8	9.3
	5×10^{-1}	"	"	12.5	12.0

Legends

1. Titration curves in terms of buffer index (β) for secretions of cells of Chlorella 7-11-05 suspended in distilled H_2O and kept in dark for time intervals indicated on the curves.

2. Titration curves in terms of buffer index (β) for secretions of cells of Chlorella 7-11-05 suspended for 44 hours in dilute HCl. Acid concentrations as indicated on the curves.

3. Titration curves in terms of buffer index (β) for secretions of cells of Chlorella 7-11-05 suspended for 44 hours in 1×10^{-3} N HCl. The amounts of cells in grams dry weight per liter of suspension are indicated on the curves.

4. Titration curves in terms of buffer index (β) for secretions of cells of Chlorella 7-11-05 suspended for 44 hours in 1×10^{-3} N HCl. Data from Fig. 3 are recalculated per gram of dry weight of cells.

5. Titration curves in terms of buffer index (β) for secretions of cells of Chlorella 7-11-05 suspended for 44 hours in dilute KOH. Concentrations of KOH are indicated on the curves.

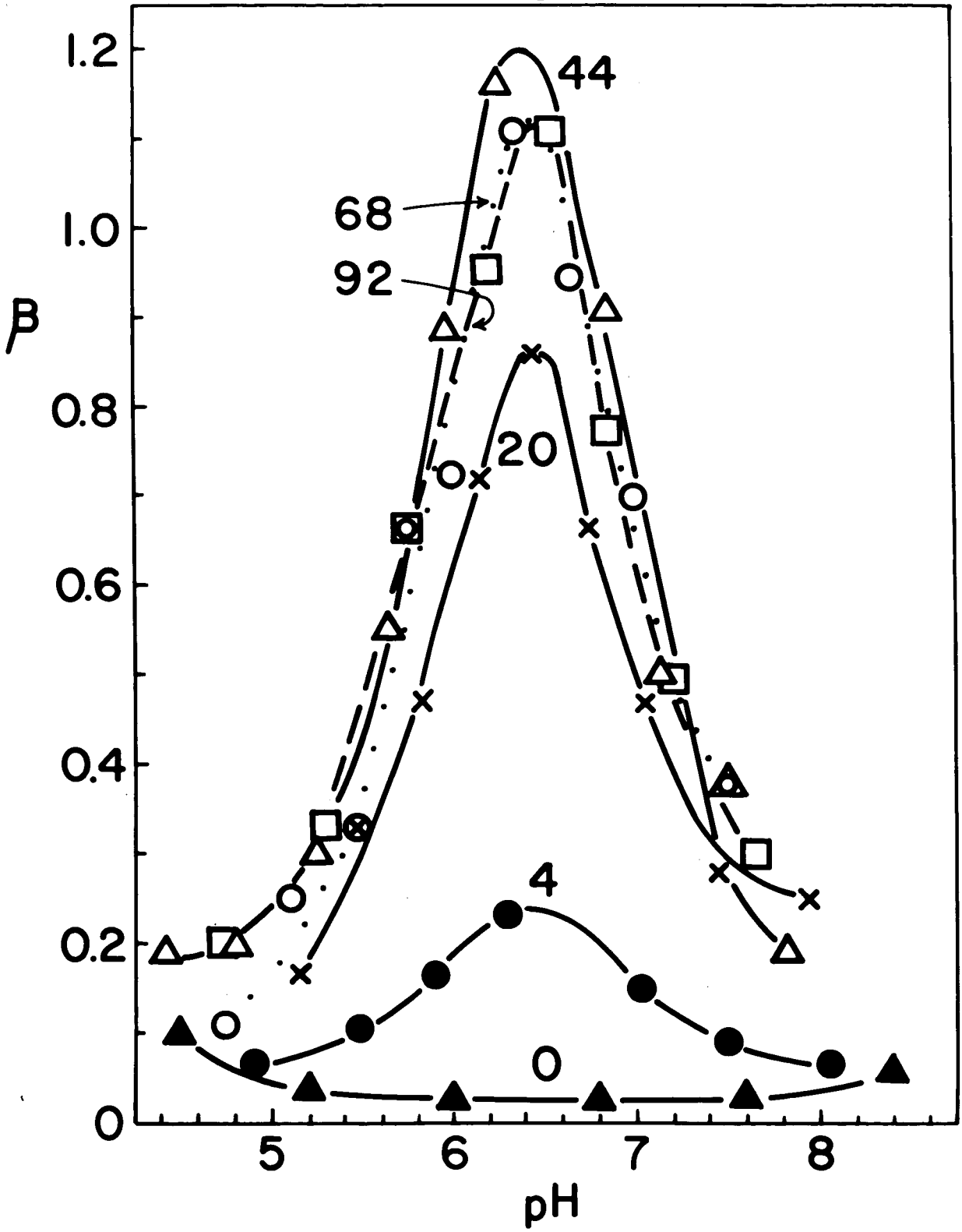
6. Titration curves in terms of buffer index (β) for secretion of cells of Chlorella 7-11-05 and for 1×10^{-3} M bicarbonate.

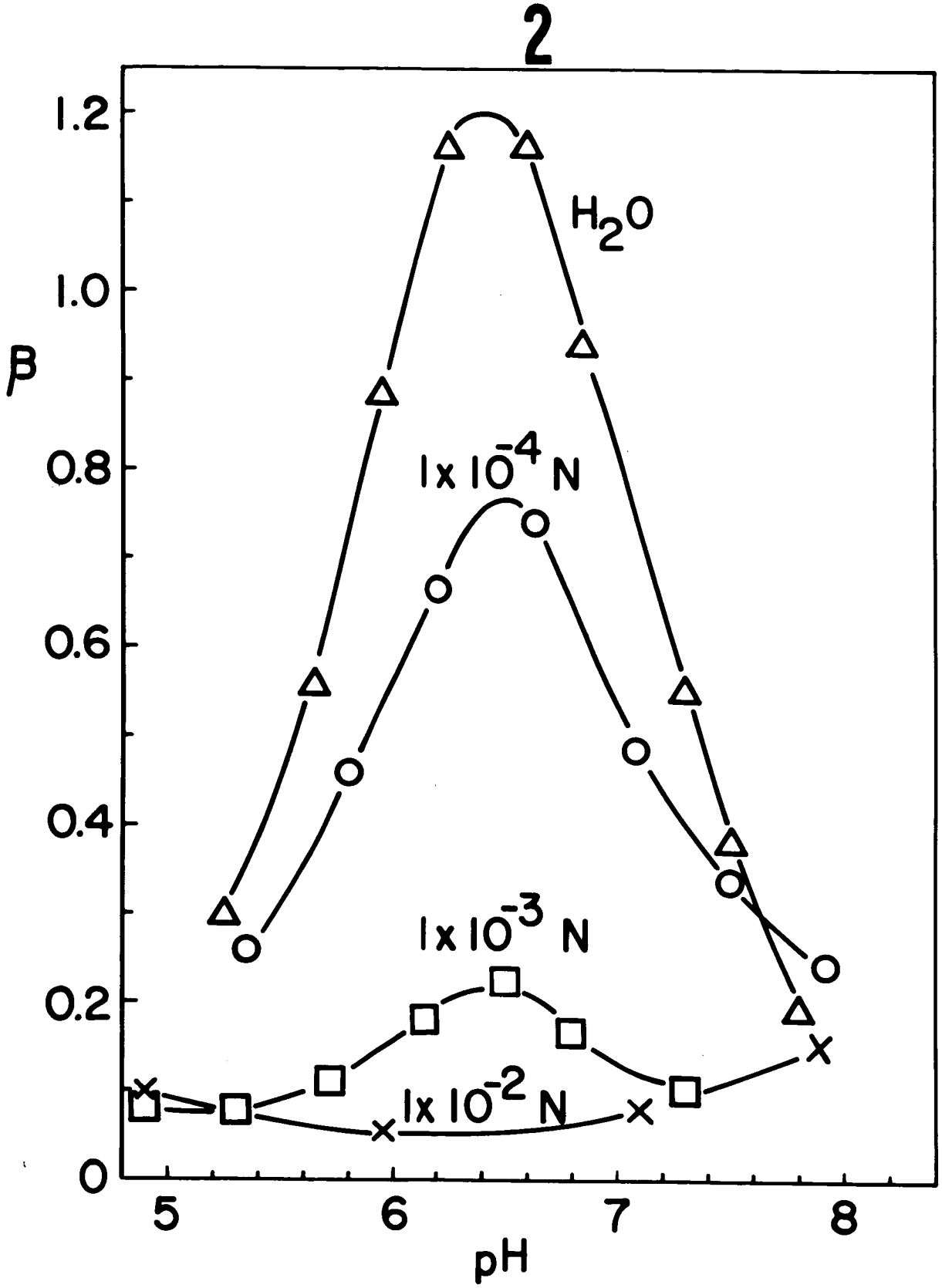
Footnotes - Page 1

1. This work was supported by funds from the National Aeronautics and Space Administration.

2. Scientific Article A1129, Contribution No. 3580 of the University of Maryland Agricultural Experiment Station.

1

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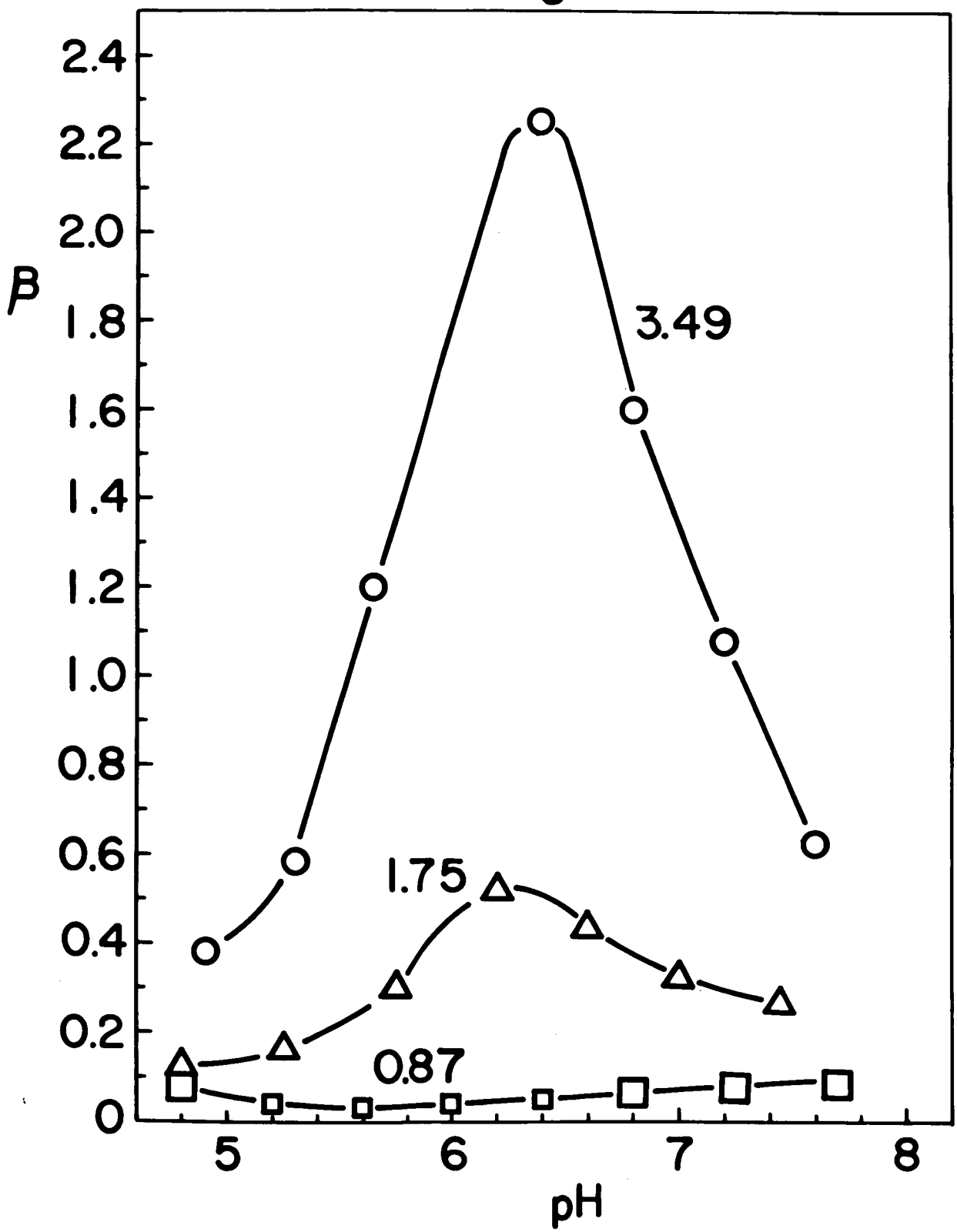
8
pH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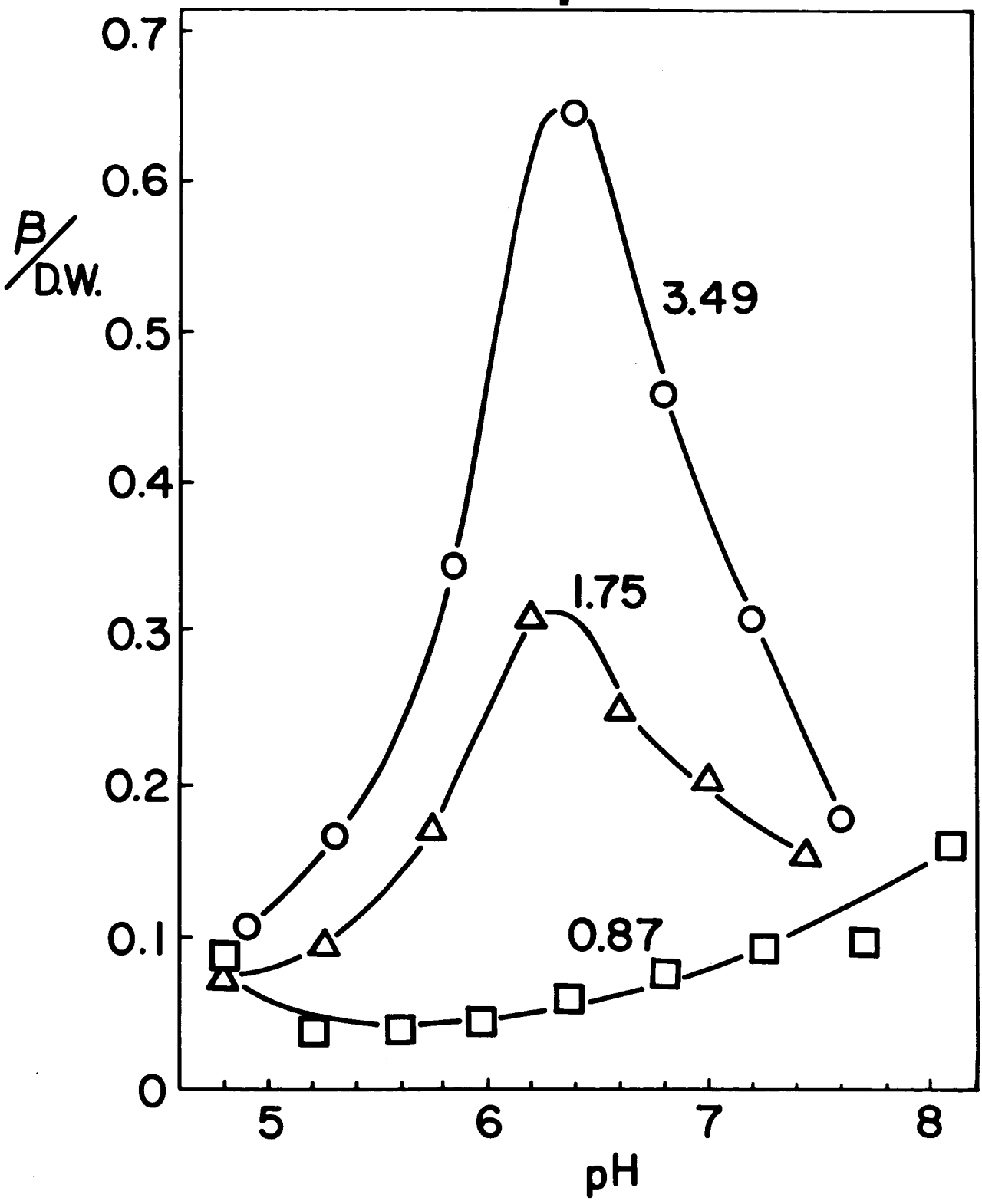

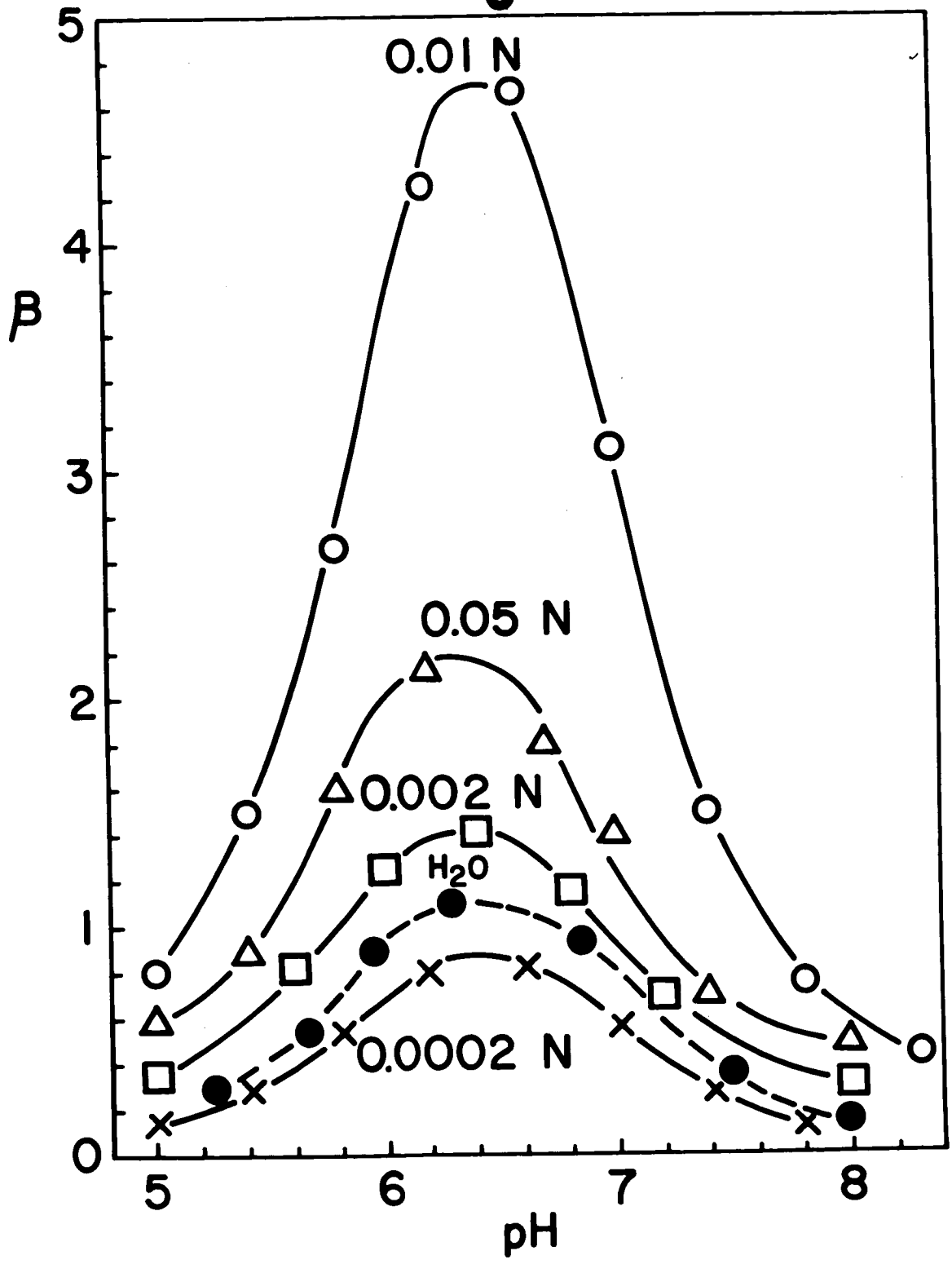
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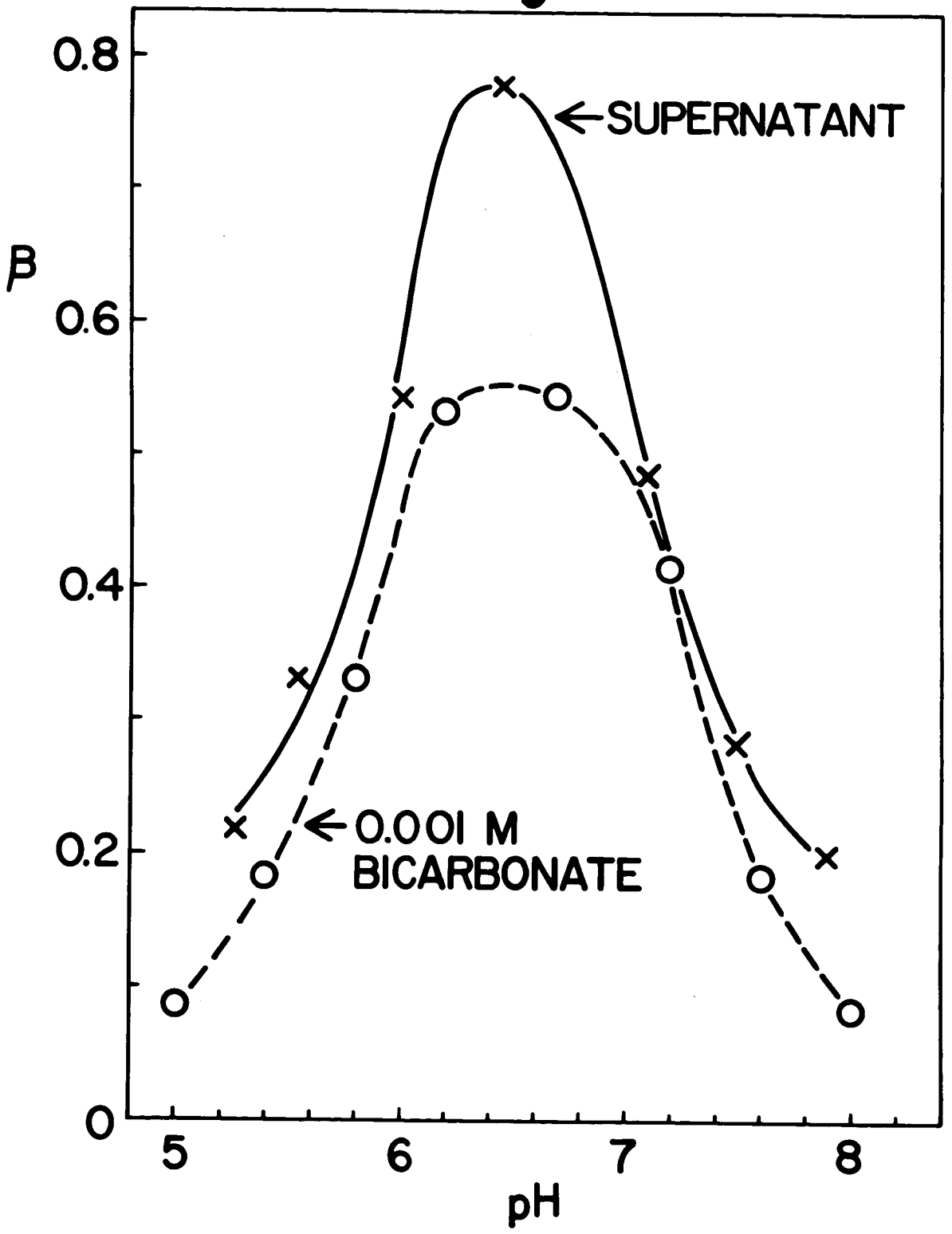
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pH

COMPARATIVE STUDIES OF STEROLS IN CHLORELLA

by

Glen W. Patterson

Thesis submitted to the Faculty of the Graduate School
of the University of Maryland in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
1964

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INTRODUCTION

Since the discovery of the therapeutic value of cortisone by Hench, et al. (1949), there has been increasing study of the occurrence and effects of natural sterols* in living systems. Widespread reports (Kritchevsky, 1958) of a relationship in humans between high blood cholesterol and atherosclerosis has led to a very intense study of the occurrence, biosynthesis, and metabolism of cholesterol and its companion sterols in higher animals. As a result, the primary biosynthetic pathways leading from acetate to cholesterol have been elucidated (Wilson, 1963), although the exact role of cholesterol in the metabolism of animals is still not understood. Much less is known about the sterols of plants. The search for a plant sterol which would be a good precursor in a commercial synthesis of cortisone yielded little knowledge of plant sterols since the interest was only in sterols which occurred in relatively high quantities in the plant. Many sterols occurring as secondary components in mixtures were overlooked because mixtures of plant sterols are notoriously difficult to separate. With the advent of modern chromatographic techniques, especially gas-liquid chromatography, these mixtures may be separated and identified, and increasing attention is being given to precise characteristics of the sterol complement of plants.

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* In this paper a sterol is understood to refer to those steroids resembling cholesterol by the presence of a hydroxyl group at C-3 and an alkyl side chain at C-17.

Sterols are widely distributed in nature. All animals and nearly all plants contain sterols, but bacteria, except for six species (Fiertel, 1959; Aaronson and Baker, 1961) and certain blue-green algae (Carter et al. 1939) appear to be free of sterols. Many species of algae, including all the algal classes, have been investigated for the presence of sterols, but in most cases the sterols were not precisely identified and were identified only as "sitosterol", which was in most cases simply a term for a non-homogenous mixture of sterols. The first algal sterol to be isolated and clearly identified was fucosterol from Fucus vesiculosus (Heilbron et al. 1934). All brown algae so far studied have contained fucosterol although some species also contain the C-20 alpha isomer, sargasterol (Tsuda et al. 1958). Red algae contain a wide variety of sterols. One of them is cholesterol (Tsuda, 1957), a fact which came as a surprise to those who classified sterols as zoosterols or phytosterols. The sterols of only five species of green algae have been clearly identified. Szabo et al. (1961) examined sixteen species of unicellular green algae and found the sterol content to vary from 0.07% to 0.51% on a dry weight basis, but the sterols present were not identified. Ergosterol, the common sterol of fungi, was found to be the principal sterol of Chlorella pyrenoidosa (Klosty and Bergmann, 1952). Scenedesmus obliquus, strain D_3, (Bergmann and Feeney, 1950) and Chlorella vulgaris (Patterson, 1963) were found to contain chondrillasterol. Tsuda and Sakai (1960) isolated haliclonasterol from Monostroma nitidium and Δ^5 avenasterol from Enteromorpha linza. Recently, Otsuka (1963) has reported the presence of ergosterol, a Δ^5 sterol, and a corticoid type compound in Chlorella ellipsoidea. Otsuka's conclusions were based on several color tests

and the ultraviolet absorption of the steroid fraction. The most common sterol of the Chlorophyta was reported to be "sitosterol" by Bergmann (1953). This supported the view that higher plants originated from the Chlorophyta since β-sitosterol is the most common sterol of higher plants. However, since the efficient separation of plant sterol mixtures became possible, no isolation of "sitosterol" has been reported from the green algae.

A new compound, 1- 4(diethylaminoethoxy)phenyl -1-(p-tolyl)-2-(p-chlorophenyl) ethanol, called triparanol, is thought to be a specific inhibitor of cholesterol biosynthesis (Blohm and MacKenzie, 1959). The exposure of Tetrahymena to triparanol (Holz et al. 1962) caused an inhibition of growth which could be reversed by the individual addition to the medium of numerous sterols and fatty acids. A combination of sterols and certain fatty acids gave better protection than the sterols cholesterol and ergosterol alone.

This paper represents the first steps in a program designed to determine the role of sterols in green algae. It reports the results of analytical determination of the sterols of six species of the genus Chlorella, and presents data concerning the effect of the substitution of various lipids in the metabolism of algae in which sterol synthesis had presumably been blocked by triparanol.

MATERIALS AND METHODS

Sterol Isolation

Cells of Chlorella protothecoides var. mannophila Shihira and Krauss and C. protothecoides var. communis Shihira and Krauss were grown in medium 1 as shown in Table I. Cells of Chlorella sorokiniana Shihira and Krauss, C. ellipsoidea Gerneck, C. nocturna Shihira and Krauss, C. vannielii Shihira and Krauss, and C. saccharophila (Krüger) Migula were grown in medium 3 which is the same as medium 2 except for the addition of 5.00 g/l of glucose and an alteration of the phosphate buffer ratios to 0.67 g/l KH_2PO_4 and 0.33 g/l K_2HPO_4 so that the final pH of the medium is about 6.5. All cells were transferred from agar slants to one-liter Erlenmeyer flasks containing 500 ml of medium. After reaching an optical density of 1.5-2.0, they were transferred aseptically to carboys closed by cotton plugs through which cotton-plugged bubbling-tubes were passed. A constant flow of air from an oil-free compressor provided oxygen for metabolism of glucose and served to keep the cells suspended. All cultures used were pure; those showing contamination were rejected. After 7-10 days of growth the cells were centrifuged in a Sharples Super-Centrifuge. The cells were frozen and stored in the freezer at -20 C. The average yield of C. vannielii and C. sorokiniana was 3 g dry weight per liter. The yield of the other Chlorella species averaged 2.0-2.5 g dry weight per liter.

The cells were thawed and enough glacial acetic acid was added to make a thin paste. This was allowed to stand at 70 C for one hour.

Table I

Organic Medium for the Heterotrophic Culture of Chlorella

Salts	Grams per Liter of Distilled Water
NH_4Cl	1.00
$MgSO_4 \cdot 7H_2O$	0.25
KH_2PO_4	0.33
K_2HPO_4	0.67
EDTA·NaFe	0.0385
EDTA·Na_2Mn	0.0071
EDTA·Na_2Ca	0.0077
EDTA·Na_2Co	0.0093
EDTA·Na_2Cu	0.0077
EDTA·Na_2Zn	0.0067
H_3BO_4	0.0057
MoO_3	0.0015
Thiamine hydrochloride	0.00001
Glucose	5.00

Table II

Inorganic Medium for the Autotrophic Culture of Chlorella

Salts	Grams per Liter of Distilled Water
KNO_3	1.00
$MgSO_4 \cdot 7H_2O$	0.25
KH_2PO_4	0.50
K_2HPO_4	0.50
EDTA·NaFe	0.0385
EDTA·Na_2Mn	0.0071
EDTA·Na_2Ca	0.0077
EDTA·Na_2Co	0.0093
EDTA·Na_2Cu	0.0077
EDTA·Na_2Zn	0.0067
H_3BO_3	0.0057
MoO_3	0.0015

The acetic acid was removed in a flash evaporator, and the cells were placed in an oven at 60 C for 24 hours. The dried cells were ground in a Wiley mill to pass a 40-mesh screen and were placed in a Soxhlet apparatus and extracted with ether, acetone and ethanol (1:1:2) for 24 hours. The extract was filtered to remove particulate matter. The solvents were removed in a flash evaporator. The lipid material was weighed and saponified for 12 hours with a 20% solution of KOH in 80% aqueous ethanol under an atmosphere of nitrogen. Approximately half of the ethanol was removed by distillation and was replaced by an equal volume of water. The lipid material was extracted with ether in a liquid-liquid extraction apparatus (Patterson, 1963). The non-saponifiable fraction was thus concentrated in the ether solution. The ether was evaporated under nitrogen, and the weight of the non-saponifiable lipid was taken. The non-saponifiable lipid was fractionated (Heftmann et al. 1960) on grade III neutral alumina in a column 3 cm in diameter. Thirty grams of alumina was required for each gram of sample fractionated. One hundred ml fractions were collected as follows: Fractions 1-2, 5%; 3-4, 10%; 5-6, 25%; 7-10, 50% benzene in Skellysolve F; 11-12 benzene; 13-14, 10%; 15-16, 50% ether in benzene; 17-18, ether; 19-20, 10% methanol in ether. The sterol was eluted in the 50% benzene-Skellysolve F fractions. The solvents were evaporated under nitrogen, and the sterol fraction was acetylated by heating on a hot-water bath for one hour with 25 ml of acetic anhydride for each 100 mg of sterol. The solution was poured into water and the precipitate recrystallized from methanol. When gas-liquid chromatographic analysis showed the sterol fraction to consist of a mixture of two or more sterols, the acetate derivatives were subjected to column chromatography on Anasil B which was added to

the 3 cm X 40 cm column in a slurry with N-hexane (Johnson et al. 1963). The column was allowed to pack by gravity. The sterol acetates were added to the column dissolved in a minimum amount of N-hexane and were eluted from the column with 2% ether in N-hexane. Fifteen ml fractions were collected on an ISCO model A fraction collector. Fractions containing steroids were located by gas chromatographic analysis. Those containing one particular steroid were combined and rechromatographed. This process was repeated as many times as was necessary for complete separation of the steroid mixture. The number of times required depended upon the relative polarity of the steroids comprising the mixture.

After hydrolyzing the acetate to the free sterol with 2% methanolic KOH, the sterol was further purified by the precipitation method of Schonheimer & Sperry (1934). A solution of 1% digitonin in ethanol was added to a solution of the crude sterol in ethanol at 60 C, precipitating all 3 β-hydroxy sterols (Windaus, 1914) from solution. After standing overnight the precipitate, or digitonide, was separated by centrifuging and decanting off the liquid. The precipitate was washed several times with ethanol and then with ether. It was dried in an oven at 70 C and its weight determined. The approximate weight of the sterol is equal to the weight of the digitonide multiplied by 0.239 (Fieser, 1959). The digitonide was split by Bergmann's (1940) modification of Schonheimer and Dam's method (1934). The digitonide was dissolved in a minimum amount of cold pyridine -- splitting the digitonide. Ether was added to precipitate the digitonin and any remaining undissociated digitonides; both were then

separated by centrifugation. Enough hot ethanol was added to the precipitate to make a 1% solution. The insoluble digitonide was separated by centrifugation, and any remaining sterol was separated again as given above. The free sterol was obtained by evaporating the ether under nitrogen and recrystallizing it several times -- first from methanol and then from acetone.

Sterol Characterization and Identification

The characterization of an unknown sterol required a number of chemical and physical tests as follows:

The Liebermann-Burchard reaction (Liebermann, 1885; Burchard, 1890) consists of adding a solution of acetic anhydride and concentrated sulfuric acid (19:1) to a few crystals of sterol dissolved in chloroform. A green colored solution is indicative of a positive sterol test, and this is now a common test for their detection and quantitative determination (Schonheimer and Sperry, 1934). This test is also of use qualitatively. Moore and Baumann (1952) have shown that by this reaction one can distinguish between a Δ^5 and a Δ^7 sterol. The maximum color intensity is reached after 30-35 minutes in the case of a Δ^5 sterol, but the more reactive Δ^7 sterol reaches maximum intensity in one to one and a half minutes. Cook (1961), however, has shown that many saturated sterols either do not respond to the test, or give a very faint color reaction.

The melting points of the sterols and their acetate derivatives were determined with a calibrated Fisher-Johns Melting Point Apparatus. The acetate derivatives were prepared as described earlier.

The determinations of optical rotation were made with a Rudolf model 70 polarimeter. Eight milligrams of sterol were dissolved in 1 ml of chloroform and placed in a 1 dm tube. The readings were taken at 25 C using a sodium light source.

Ultraviolet absorption-spectra of cyclic dienes fall into two groups depending on whether the double bonds are in the same or different rings (Fieser, 1937). Cyclic monoenes have no ultraviolet absorption above 220 mμ. Ultraviolet spectra were determined with a Perkin-Elmer recording spectrophotometer using a solution of sterol in 95% ethanol.

The infrared spectrum of steroids aids in identification of double bonds, carbonyl groups, and hydroxyl groups. More specific information of the structure of a steroid may be obtained by examination of the absorption in the region below 1350 cm^{-1}. The samples were dissolved in CS_2 and determined in a Perkin-Elmer infrared spectrophotometer.

A Glowall Chromalab, Model A-110, dual column chromatograph was used for qualitative and quantitative analysis of the sterols isolated. The three column packings and operating conditions are as follows: 1% QF-1 on 100-120 mesh Gas Chrom P, column temperature 217 C, detector 235 C, flash heater 260 C; 3% SE-52 on 100-120 mesh Gas Chrom P, column temperature 240 C, detector 245 C, flash heater 260 C; 1% Diethylene Glycol Succinate on 100-120 mesh Gas Chrom P, column temperature 205 C, detector 225 C, flash heater 260 C. All columns were glass coiled, 1.8 m long and 3.4 mm inside diameter. The chromatograph employed an Aerograph Model 650 hydrogen generator to supply hydrogen and oxygen for the flame ionization detector. The carrier gas was argon at 20 PSI. A Leeds and Northrup Model #40 Speedomax recorder with a range of 50-0 MV was used to trace the

results. Samples were injected from a Hamilton 1 μl syringe. Retention times are reported relative to cholestane.

Triparanol Inhibition Studies

Pure cultures of Chlorella sorokiniana Shihira and Krauss were employed in the study of the inhibitory action of triparanol. They were maintained on agar slants, and, prior to experiments, were subcultured several times in liquid medium until optimal, exponential growth was obtained. Inocula for all experiments were taken from cultures which were in the logarithmic phase of growth. Cells were grown in medium 2 shown in Table II. The initial pH value of the medium was 7.0. Cultures were grown in Pyrex test-tubes, 18 mm in diameter and 150 mm long. The tubes were closed with cotton plugs through which cotton-plugged bubbling-tubes were passed. A constant flow of a 1% CO_2-in-air mixture served to provide CO_2 and to keep the cells in suspension. The algae were maintained at their maximum growth rates by placing the test-tubes in a Lucite water bath maintained at 25 C. The cells were illuminated by two banks of 40-watt General Electric fluorescent lamps giving an illuminance of 800 ft-c, as measured by a Weston Illumination Meter at the surface of the test-tubes.

Since triparanol itself is almost insoluble in water, a water-soluble form, triparanol succinate was employed. A triparanol succinate stock solution was prepared to contain 0.6 mg in 10 ml of water. An inhibitor concentration of 1×10^{-6} molar was obtained by adding 0.085 ml of the stock solution to a test tube containing a final volume of 10.0 ml. The triparanol succinate stock solution was prepared immediately prior to each experiment. Fatty acids, sterols, and complex lipid mixtures

were added to the medium as described by Holz et al. (1962). Fatty acids and complex lipids were dissolved in ether and added to the test tubes before the medium of the inhibitor. The ether was evaporated leaving the dissolved materials in a thin film on the bottom of the culture tube. A sterol stock solution was prepared by dissolving 4 mg of sterol in 10 ml of propylene glycol. A final sterol concentration of 5 μ molar was obtained by adding 0.05 ml of the stock solution to the culture tube containing a final volume of 10.0 ml. All growth experiments were conducted under aseptic conditions. Cells from an actively growing culture were transferred to the test solution to give an initial optical density of 0.02. Growth was recorded by measuring optical density in the culture tubes at 550 mμ on a Coleman, Jr. Spectrophotometer. Experiments were carried out in duplicate.

RESULTS AND DISCUSSION

Triparanol succinate proved to be a very effective growth inhibitor of C. sorokiniana. Little or no algal growth occurred at triparanol concentrations greater than 2×10^{-6} molar. Cells from cultures containing strongly inhibitory concentrations of triparanol examined microscopically did not appear to differ markedly from normal cells. The average size of treated cells was slightly larger and the pigment concentration appeared to be less than that of normal cells. Although Holz et al. (1962) and Aaronson et al. (1962) have reported the reversal of triparanol inhibition by sterols, fatty acids, and complex lipids in the ciliates Tetrahymena and Ochromonas, only oleic acid of the fatty acids and none of the sterols reversed the inhibition of triparanol. Results are shown in Table III. The ability of the tweens to effectively reverse the action of triparanol is probably due to the large amount of oleic acid present in tween 80 in the ester form and the presence of oleic or other unsaturated fatty acids in tweens 20, 40, and 60. The results obtained here are different from those of Holz or Aaronson. Holz postulated that the primary point of inhibition in Tetrahymena is the reduction of the C-24 sterol double bond with a secondary inhibition in fatty acid biosynthesis. The primary site of inhibition in Ochromonas is thought to be in unsaturated fatty acid biosynthesis with secondary inhibition at the mevalonic acid stage of sterol biosynthesis. In Chlorella triparanol does seem to affect unsaturated fatty acid metabolism because of the removal of triparanol inhibition by the unsaturated oleic acid, but no evidence of inhibition of sterol biosynthesis could be found.

Table III

The Protection Afforded by Various Lipids Against Triparanol Succinate Inhibition of Growth of *Chlorella sorokiniana* Shihira and Krauss

Lipids	Optical Density after 24 Hours* μM Triparanol Succinate		
	0	2.5	5
Control	0.31	0.03	0.02
palmitic acid (.05 mM)	0.27	0.03	0.02
palmitic acid (.10 mM)	0.25	0.05	0.02
myristic acid (.05 mM)	0.27	0.03	0.02
stearic acid (.10 mM)	0.25	0.05	0.02
oleic acid (.025 mM)	0.31	0.10	0.05
oleic acid (.05 mM)	0.31	0.16	0.11
oleic acid (.10 mM)	0.31	0.31	0.24
cholesterol (2 ppm)	0.31	0.03	0.02
ergosterol (2 ppm)	0.31	0.03	0.02
chondrillasterol (2 ppm)	0.30	0.03	0.02
tween 20 (5 ppm)	0.31	0.18	0.14
tween 20 (10 ppm)	0.31	0.18	0.10
tween 40 (5 ppm)	0.31	0.25	0.22
tween 40 (10 ppm)	0.31	0.21	0.20
tween 60 (5 ppm)	0.31	0.26	0.22
tween 60 (10 ppm)	0.31	0.26	0.24
tween 80 (5 ppm)	0.31	0.31	0.31
tween 80 (10 ppm)	0.31	0.30	0.28

* Initial Optical Density = 0.02

Data from the lipid extraction of seven Chlorella species are shown in Table IV. It must be noted that the culture conditions under which the cells were grown were not necessarily optimal for either lipid or sterol production. Cells grown in medium 3 but cultured in a commercial fermentor with rapid stirring and strong aeration contained twice as much sterol as cells grown in carboys. The actual sterol yield from cells growing under optimal conditions is presumed to be considerably higher than the data in Table IV might indicate.

The principal sterol from C. vannielii, formerly known as C. pyrenoidosa (Shihira and Krauss, 1964), is ergosterol (Klosty and Bergmann, 1952). Gas chromatographic analysis of the crude sterol fraction from C. vannielii showed the presence of a second sterol. Its relative retention time on the three columns shown in Table V was identical to that of 7-dehydrocampesterol and 22-dihydroergosterol. These compounds differ from each other only in the orientation of the C-24 methyl group (Figure 1). The algal sterols were separated on a silica gel column; the minor sterol being eluted first. The ultraviolet absorption spectrum of the unknown had the characteristics of a $\Delta^{5,7}$ diene, with its absorption maximum at 282 mμ. It gave a rapid positive Liebermann-Burchard color test indicative of a sterol with a Δ^{7} double bond. The lack of a strong infrared absorption band at 10.3 μ is further evidence that the compound does not contain a Δ^{22} double bond (Jones, 1950; Turnbull, et al. 1950). The melting point of the unknown is quite near that of 22-dihydroergosterol but considerably lower than that of 7-dehydrocampesterol (Table VI). Thus, the data indicate that the secondary sterol of C. vannielii is 22-dihydroergosterol. Its presence has not been previously demonstrated

Table IV

The Sterols and Unsaponifiable Lipids

of Seven *Chlorella* Species

Organism	% Dry Weight		
	Lipid	Unsaponifiable Lipid	Sterol
Chlorella sorokiniana Shihira and Krauss	20	3.5	0.16
Chlorella nocturna Shihira and Krauss	26	3.1	0.20
Chlorella protothecoides var. *mannophila* Shihira and Krauss	14	1.6	0.13
Chlorella ellipsoidea Gerneck	10	0.8	0.10
Chlorella saccharophila (Krüger) Migula	20	1.3	0.17
Chlorella vannielii Shihira and Krauss	20	2.2	0.20
*Chlorella vulgaris** Beyerinck	14	1.3	0.13

* Patterson, 1963

Table V

A Comparison of the Retention Times on Gas Chromatographic Columns of Certain Known Sterols With Those Sterols Isolated From Chlorella

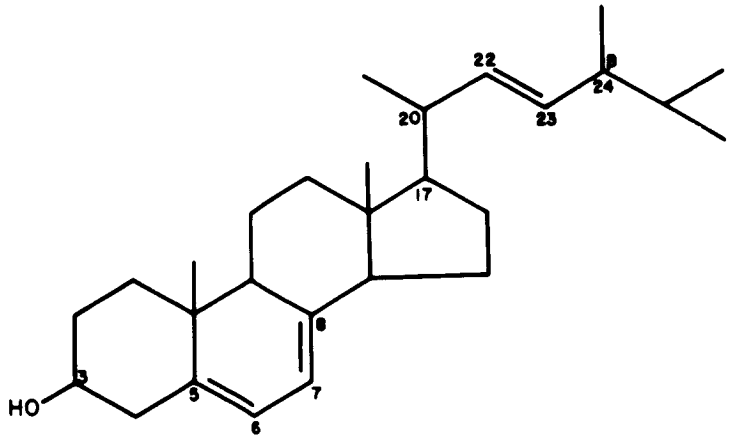
Compounds	% of Total Algal Sterols	Relative Retention Time[a]		
		SE-52[b]	QF-1[c]	DEGS[d]
ergosterol		2.44	3.50	14.9
22-dihydroergosterol		2.82	4.19	17.6
7-dehydrocampesterol		2.82	4.19	17.6
haliclonasterol		2.55[e]	3.75[e]	12.5[e]
campesterol		2.55	3.75	12.5
22-dihydrobrassicasterol		2.55	3.75	12.5
stigmasterol		2.80	3.82	12.7
poriferasterol		2.80	3.82	12.7
β-sitosterol		3.20	4.59	14.8
clionasterol		3.20	4.59	14.8
C. vannielii Shihira and Krauss				
Sterol #1	76	2.44	3.50	14.9
Sterol #2	24	2.81	4.18	17.3
C. sorokiniana Shihira and Krauss				
Sterol #1	75	2.45	3.49	14.8
Sterol #2	25	2.81	4.18	17.4
C. nocturna Shihira and Krauss				
Sterol #1	66	2.44	3.49	14.7
Sterol #2	34	2.83	4.19	17.5
C. ellipsoidea Gerneck				
Sterol #1	28	2.54	3.76	12.2
Sterol #2	56	2.80	3.82	12.7
Sterol #3	16	3.19	4.56	14.8

Table V continued

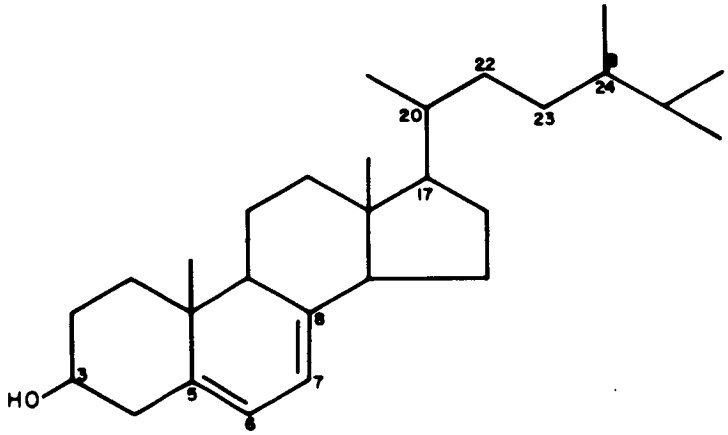
Compounds	% of Total Algal Sterols	Relative Retention Time[a]		
		SE-52[b]	QF-1[c]	DEGS[d]
C. saccharophila (Krüger) Migula				
Sterol #1	30	2.54	3.75	12.4
Sterol #2	60	2.80	3.82	12.6
Sterol #3	7	3.20	4.57	14.8
Sterol #4	3			
C. protothecoides var. mannophila Shihira and Krauss				
Sterol #1	62	2.19	3.11	10.3
Sterol #2	16	2.49	3.59	11.9
Sterol #3	22	2.89		14.1
C. protothecoides var. communis Shihira and Krauss				
Sterol #1	8	2.23	3.09	10.2
Sterol #2	35	2.50	3.50	11.9
Sterol #3	57	2.95	4.24	14.2

a - relative to cholestane.
b - column 1.8 m x 3.4 mm I.D., 3% SE-52 on 100-120 mesh Gas Chrom P, 20 p.s.i., 240 C, cholestane time 9 minutes.
c - column 1.8 m x 3.4 mm I.D., 1% QF-1 on 100-120 mesh Gas Chrom P, 20 p.s.i., 217 C, cholestane time 4 minutes.
d - column 1.8 m x 3.4 mm I.D., 1% diethylene glucol succinate on 100-120 mesh Gas Chrom P, 20 p.s.i., 205 C, cholestane time 2 minutes.
e - putative values based on campesterol.

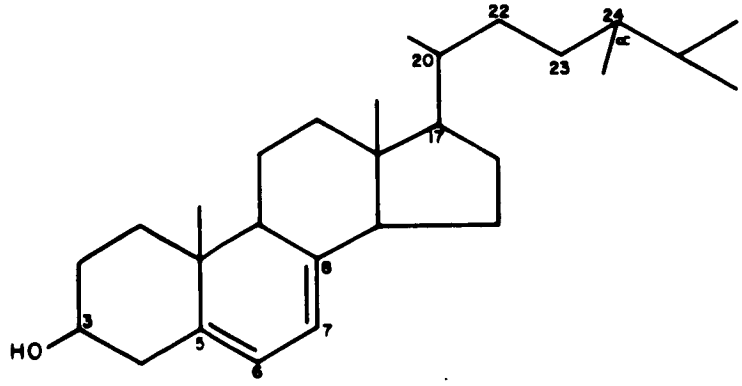
Figure 1: Structural formulae for ergosterol, 22-dihydro-ergosterol, and 7-dehydrocampesterol.



ERGOSTEROL



22-DIHYDROERGOSTEROL



7-DEHYDROCAMPESTEROL

Table VI

Optical Rotation, Melting Point, and Ultraviolet Absorption Data of *Chlorella* Sterols Compared to Those of Known Sterols

Sterol	UV Absorption at 282 mμ	Specific Rotation	M.P. (Sterol)	M.P. (Acetate)
C. vannielii sterol #2	yes	-	149-152	-
22-dihydroergosterol[a]	yes	-109	153	158
7-dehydrocampesterol[a]	yes	-109	165	-
C. saccharophila sterol #1	no	-	-	142-146
C. ellipsoidea sterol #1	no	-42	159-160	146-148
22-dihydrobrassicasterol[b]	no	-46	158	145
campesterol[c]	no	-34	163-164	141
haliclonasterol[d]	no	-42	141	141
C. saccharophila sterol #2	no	-	-	146-148
C. ellipsoidea sterol #2	no	-55	156-157	147-148
poriferasterol[a]	no	-49	156	147
stigmasterol[a]	no	-46	170	144
C. saccharophila sterol #3	no	-	-	135-140
C. ellipsoidea sterol #3	no	-	143-144	139-141
clionasterol[e]	no	-42	140	140
β-sitosterol[a]	no	-37	137	127

a-Bergmann, 1960
b-Ferholz and Ruigh, 1940
c-Thompson *et al.*, 1962
d-Tsuda and Sakai, 1960
e-Bergmann *et al.*, 1950

in nature although its isolation has been expected for some time (Bergmann, 1960). In the plant kingdom the Δ^{22} double bond is a common one which frequently occurs with the 22-dihydrosterol (Bergmann, 1953).

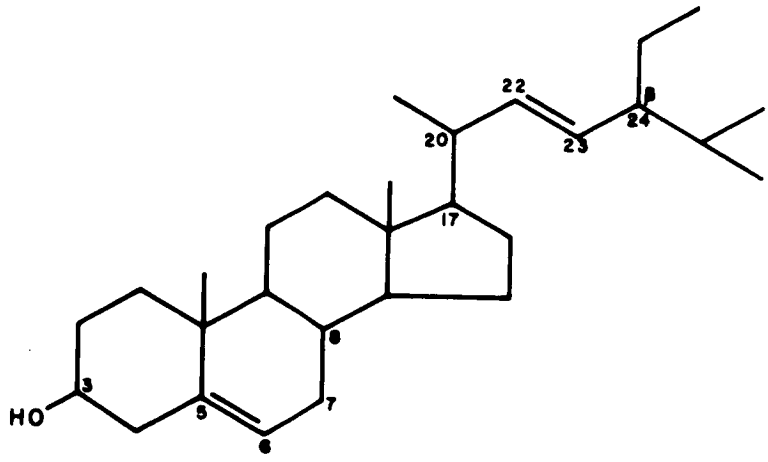
The sterols isolated from C. sorokiniana appeared identical to those of C. vannielii on gas chromatographic analysis. The minor sterol was again eluted first on separation with silica gel column chromatography. Both sterols gave rapid, positive Liebermann-Burchard color tests, and produced the typical $\Delta^{5,7}$ ultraviolet absorption curve. Therefore, the major and minor sterols of C. sorokiniana were concluded to be ergosterol and 22-dihydroergosterol, respectively.

The sterols from C. nocturna gave the same color tests and ultraviolet spectra as those from C. vannielii and C. sorokiniana. The two sterols present also had identical gas chromatographic retention times to the sterols of the above two species. The only observed difference in composition of C. nocturna sterols was a relatively higher proportion of 22-dihydroergosterol in the sterol mixture of this species (Table V). Quantitative estimations of sterol content were made by measuring the area under each peak on the gas chromatogram.

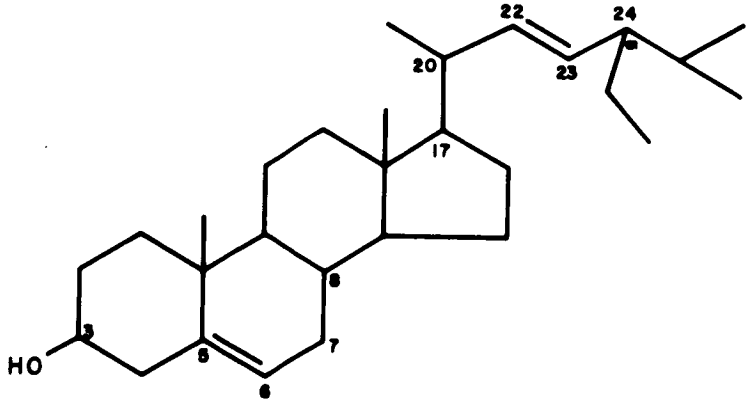
The crude sterol fraction from C. ellipsoidea failed to show any ultraviolet absorption above 240 mμ. This indicated the absence of any $\Delta^{5,7}$ compounds in the mixture. Gas-liquid chromatography indicated the presence of three sterols. These sterols were numbered in the order of their elution from the gas chromatographic columns. The sterol acetates eluted in the following order from column chromatography: #3, #1, #2. Sterols #1 and #2 were more easily separated by the column than #1 and #3. All three sterols gave slow, positive Liebermann-Burchard reactions indicative of the absence of any Δ^{7} compounds.

The #2 sterol made up 56% of the total sterol and was the least difficult to obtain in pure form. Its retention time on all gas chromatographic columns (Table V) was identical to that of stigmasterol and poriferasterol (Figure 2). Thompson et al. (1963) have shown that compounds differing only in their configuration at C-24 cannot be separated with the gas chromatographic columns presently available. Infrared absorption analysis confirmed the presence of a Δ^{22} double bond with a strong absorption band at 10.3 μ (Figure 3). The absorption in the region below 1350 cm^{-1} was nearly identical to that of stigmasterol. The unknown sterol had an optical rotation of -55 degrees which is in agreement with Bergmann's system of classification (Bergmann, 1953) according to optical rotation. According to this system, $\Delta^{5,22}$ sterols have a specific optical rotation from -50 degrees to -70 degrees. The optical rotation of the unknown compound is in close agreement with both stigmasterol and poriferasterol (Table VI). The value given for poriferasterol must be regarded as in error in the positive direction since it has always occurred in mixtures with a less levorotatory sterol, clionasterol (Valentine and Bergmann, 1941), which at that time was not completely separable. The melting points of the unknown compound and its acetate agreed quite closely with that of poriferasterol (Table VI), but the melting point of the unknown sterol was significantly lower than that of stigmasterol, while its acetate derivative melted slightly higher than stigmasterol acetate. The unknown compound must be regarded as a 24 β-ethyl-22-dehydrocholesterol, or poriferasterol.

The gas chromatographic retention times (Table V) of sterol #1 of C. ellipsoidea are identical to those of campesterol and 22-dihydro-



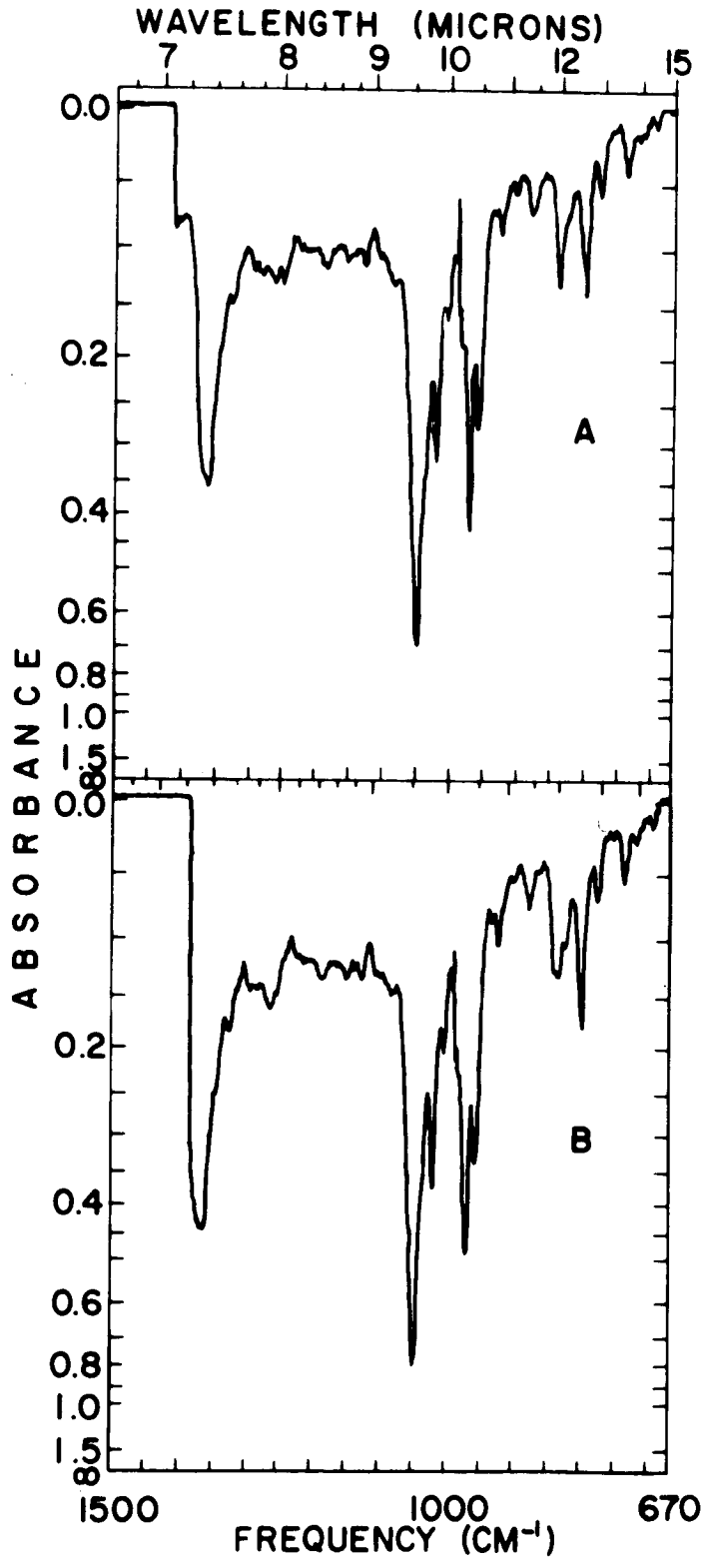
PORIFERASTEROL



STIGMASTEROL

Figure 2: Structural formulae for poriferasterol, and stigmasterol.

Figure 3: A comparison of the infrared spectrum of stigmasterol (A) with that of the #2 sterol of C. ellipsoidea Gerneck (B). Spectra were obtained from a 0.06 M solution in CS_2.


WAVELENGTH (MICRONS)
7
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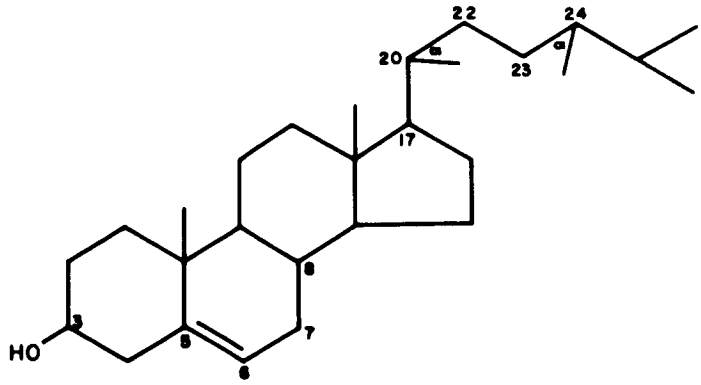
12
15
0.0
0.2
0.4
0.6
0.8
1.0
1.5
A
B
ABSORBANCE
1500
1000
670
FREQUENCY (CM⁻¹)

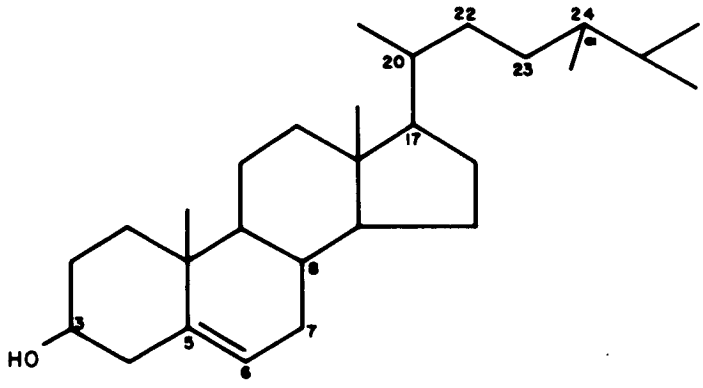
brassicasterol. These are 28-carbon sterols differing only in the configuration of the methyl group on C-24 (Figure 4). Another side chain isomer of campesterol, haliclonasterol, is assumed to have the same retention times, although none of this compound was available. The optical rotation of -42^{o} is in the region expected for a compound with a Δ^5 double bond. Recently, highly purified samples of campesterol (Thompson et al. 1962) have been no more levorotatory than -34^{o}. The more negative rotation of the unknown is significant since gas-liquid chromatography proved the absence of the more levorotatory $\Delta^{5,22}$ sterol of the algal mixture. Although the optical rotation of haliclonasterol is near that of the unknown, its melting point and that of its acetate (Table VI) are so far removed from that of the unknown that it is reasonably certain that the two compounds are not identical. It is difficult to distinguish between 22-dihydrobrassicasterol and campesterol. Infrared spectra of compounds such as these are usually indistinguishable (Dobriner et al. 1953). The infrared spectra of the unknown and campesterol are identical as seen in Figure 5. The melting points of the unknown sterol and its acetate are in better agreement with those of 22-dihydrobrassicasterol than with those of campesterol. Although 22-dihydrobrassicasterol has not been reported to occur in nature, its isolation has been anticipated (Bergmann, 1960). In impure samples it could easily have been previously identified as campesterol. Although the evidence at this point is not conclusive, it does favor the hypothesis that the #1 sterol of C. ellipsoidea is 22-dihydrobrassicasterol.

Gas chromatographic retention times of the #3 sterol of C. ellipsoidea were identical to those of β-sitosterol and clionasterol.

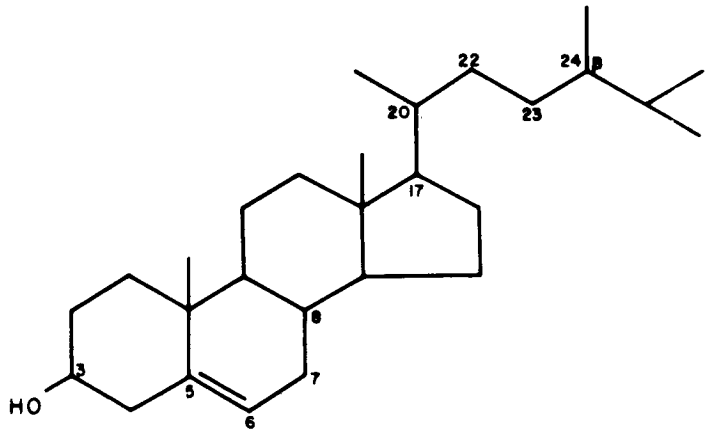
Figure 4: Structural formulae for haliclonasterol, campesterol, and 22-dihydrobrassicasterol.



HALICLONASTEROL

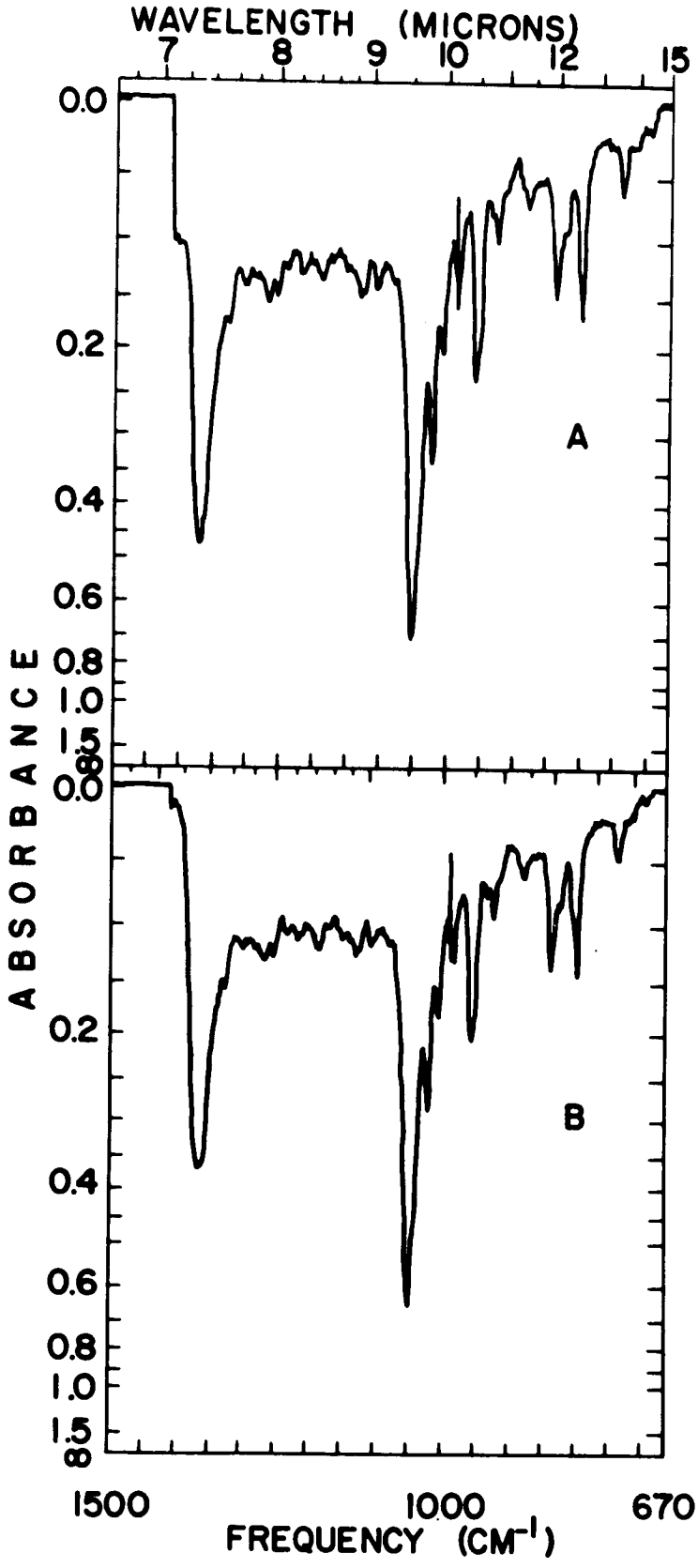


CAMPESTEROL



22-DIHYDROBRASSICASTEROL

Figure 5: A comparison of the infrared spectrum of campesterol (A) with that of the #1 sterol of C. ellipsoidea Gerneck. Spectra were obtained from a 0.06 M solution in CS_2.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ABSORBANCE
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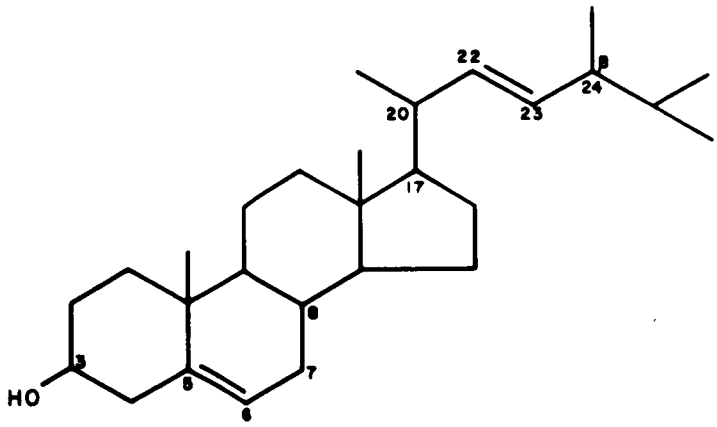
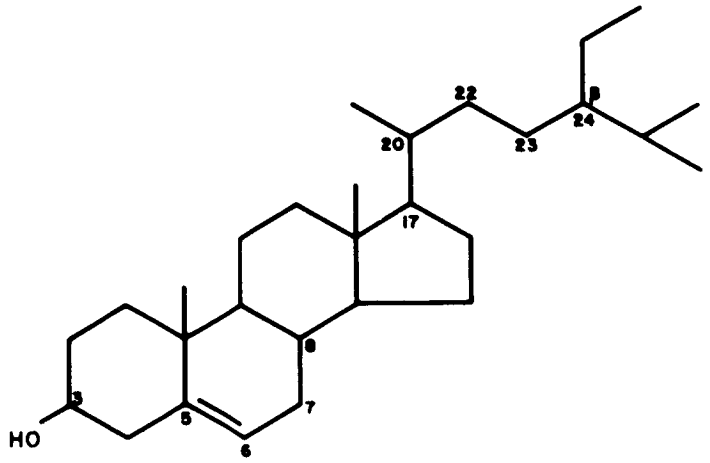
FREQUENCY (CM⁻¹)

These two sterols differ only in the configuration of the ethyl group at C-24 (Figure 6). Clionasterol is a common sterol in sponges (Bergmann, 1949) and β-sitosterol is the most common sterol of higher plants. The infrared spectra of β-sitosterol and the unknown sterol were identical (Figure 7). The melting points of the unknown sterol and its acetate were significantly higher than that of β-sitosterol and the optical rotation less levorotatory. A pure sample of clionasterol has never been isolated. Although clionasterol has been frequently isolated from sponges, it was always mixed with other sterols, usually poriferasterol. Therefore, the optical rotation given for it is probably too levorotatory, and the melting points are not to be trusted. The melting points of the unknown sterol and its acetate are close to those given for clionasterol, and the combination of physical data strongly suggest that the unknown algal sterol is clionasterol. The occurrence of poriferasterol in the same organism further suggests the presence of clionasterol, since they almost always occur together as stigmasterol and β-sitosterol do in higher plants. The hypothesis of Castle et al. (1963) that methionine is the donor of both carbon atoms of the ethyl group of β-sitosterol, and confirmation of this hypothesis by Bader et al. (1964), sheds further light on this subject. The present theory regarding side-chain biosynthesis is outlined in Figure 8. Campesterol, β-sitosterol, and stigmasterol are the most common sterols of higher plants, nearly always occurring together. If the stereochemistry of the reduction of compound IV is the same as that of compound I, then the sterols synthesized will be campesterol (III), and β-sitosterol (VI). Bennett et al. (1963) as a result of tracer experiments, have suggested that stigmasterol is formed by the dehydro-

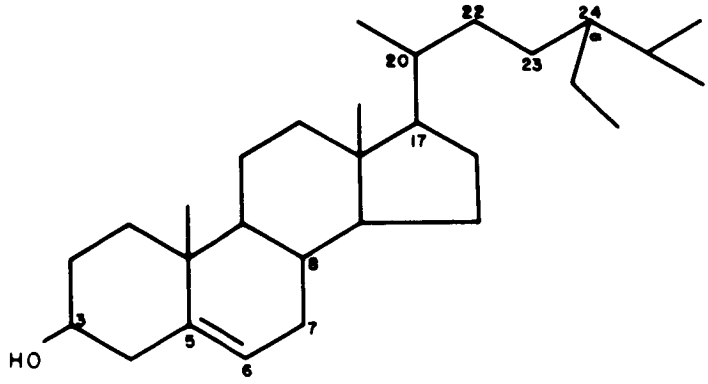
Figure 6: Structural formulae for clionasterol, brassicasterol, and β-sitosterol.



BRASSICASTEROL

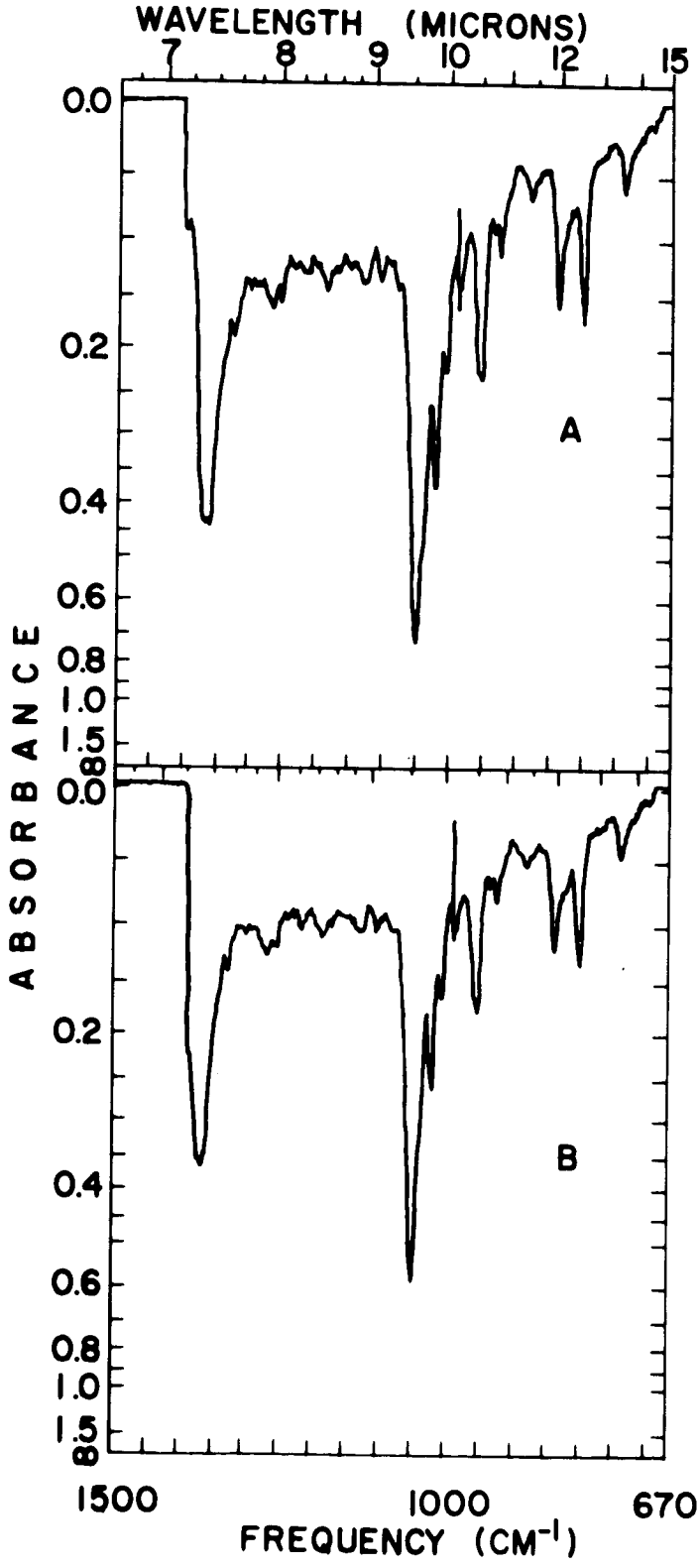


CLIONASTEROL



β - SITOSTEROL

Figure 7: A comparison of the infrared spectrum of β-sitosterol (A) with that of the #3 sterol of C. ellipsoidea Gerneck. Spectra were obtained from a 0.06 M solution in CS_2.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1.5
2.0
A
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ABSORBANCE
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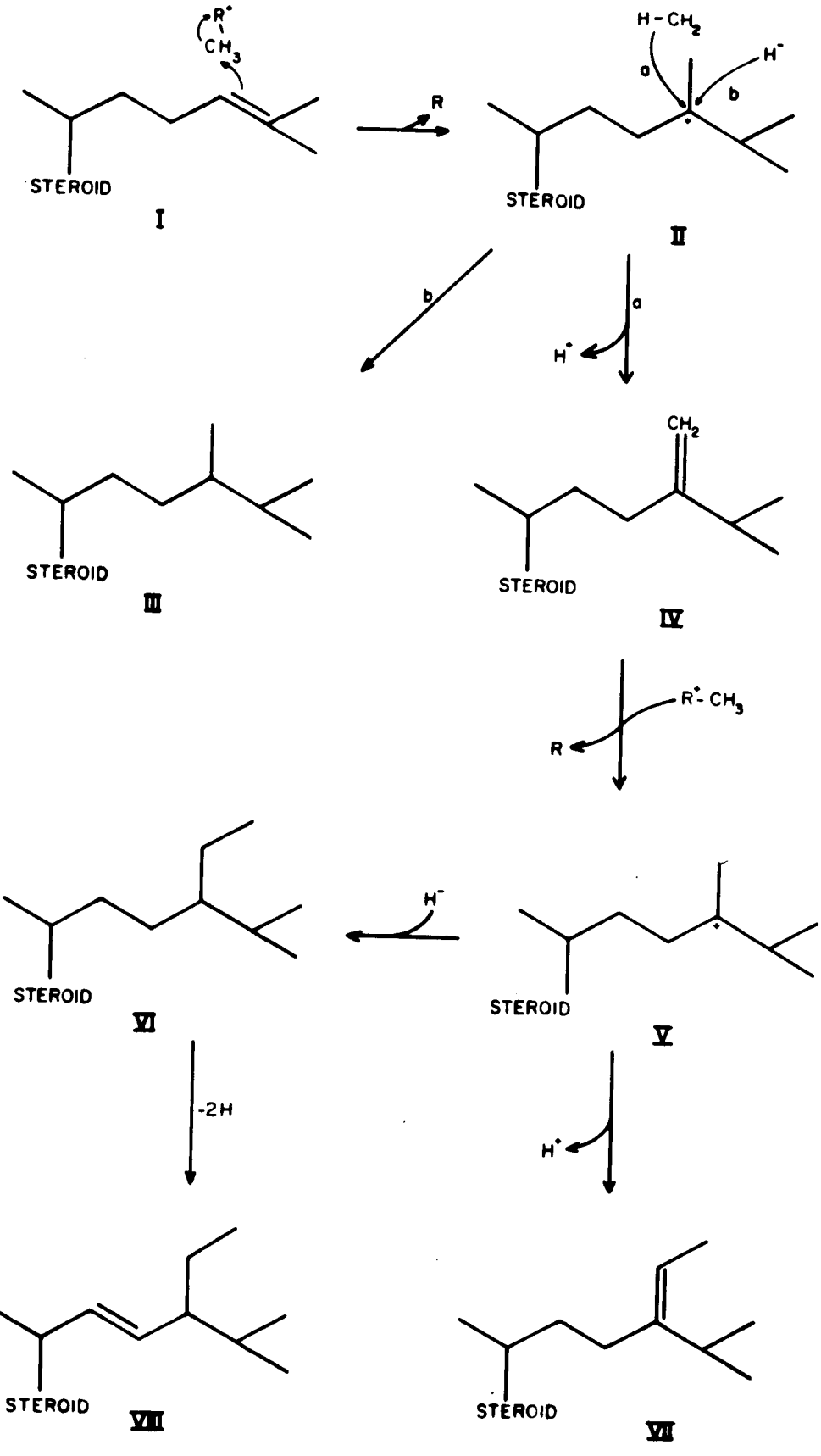
1000
670
FREQUENCY (CM⁻¹)

Figure 8: The present theory regarding transmethylation from methionine to C-24 of a sterol side chain.


R·
CH3
STEROID
I
R
H-CH2
a
H⁻
b
STEROID
II
b
a
H·
STEROID
III
CH2
STEROID
IV
R·-CH3
R
H⁻
STEROID
VI
STEROID
V
-2H
H·
STEROID
VIII
STEROID
VII

genation of β-sitosterol. Thus, there seems to be a great similarity between the system postulated for higher plants and the one which appears to exist in C. ellipsoidea. The stereochemistry of the reduction is opposite in the two systems; the C. ellipsoidea sterols containing C-24 β ethyl and methyl groups, while those in higher plants contain C-24 α ethyl and methyl groups (Tsuda et al. 1960). With the occurrence of these sterols in C. ellipsoidea, it is possible that marine invertebrate sterols are of a dietary origin just as are those of insects (Clayton, 1964).

The sterols of C. saccharophila were quite similar to those of C. ellipsoidea. The gas chromatographic data and the melting point of sterols #1, #2, and #3 indicated that they were identical to the corresponding sterols in C. ellipsoidea. The sterols were eluted in the same order and separated to the same degree as those in C. ellipsoidea. The crude sterol fraction of C. saccharophila had absorption in the 280 mμ region showing the presence of a $\Delta^{5,7}$ sterol. The absorption was traced to sterol #4 which was also the last sterol to be eluted from the silica gel column. Although not enough material was available for complete characterization, gas chromatographic data suggest that the sterol may be 22-dihydroergosterol.

The sterol mixtures occurring in C. protothecoides var. communis and C. protothecoides var. mannophila were similar to each other but not to those of any other Chlorella studied. Each of the above strains appeared to contain three sterols which behaved similarly on gas chromatography. The relative concentrations were drastically different in the two strains (Table V). The crude sterol mixture of both organisms gave ultraviolet absorption at 282 mμ indicative of the

presence of $\Delta^{5,7}$ sterols. The presence of ergosterol and 22-dihydro-ergosterol may immediately be suspected, but until the sterol mixture is separated by column chromatography no identification can be made. The short gas chromatographic retention time of the #1 sterol of both strains is evidence that it is different from any sterol yet studied in Chlorella.

SUMMARY

The characteristics of the sterols naturally occurring in six species of Chlorella were examined. The algae were grown heterotrophically on glucose. Sterols were extracted and isolated from the lipid fraction and were characterized by means of chemical and physical tests.

Chlorella vannielii, C. nocturna, and C. sorokiniana were found to contain ergosterol as their principal sterol. In addition they contained 25% as much of another sterol identified as 22-dihydroergosterol. This sterol has not previously been isolated from natural sources.

Chlorella ellipsoidea and C. saccharophila contained a sterol mixture similar to that of higher plants. These algae contained β-oriented alkyl groups at C-24 in contrast to the α-oriented groups commonly found in higher plants. Poriferasterol was identified as the principal sterol of both plants. Clionasterol and 22-dihydrobrassicasterol were tentatively identified as the two secondary sterols present. None of these sterols have previously been reported to occur in plants. The isolation of 22-dihydrobrassicasterol has not been previously reported from natural sources.

Gas chromatographic data indicated that the sterols of C. prototheocoides differed from those of the Chlorella species previously studied, but complete characterization of the sterols of the two varieties was not achieved.

In an attempt to determine the role of these sterols, cells of Chlorella sorokiniana were grown autotrophically in the presence of triparanol concentrations which were strongly inhibitory to growth. Different lipids and lipid mixtures, including sterols isolated from the algae, were added to the medium in an attempt to reverse the inhibition of triparanol. All Tween mixtures tested gave good reversal of triparanol inhibition, but oleic acid was the only pure compound which was effective. These data support the hypothesis that one site of triparanol inhibition is unsaturated fatty acid biosynthesis, but antagonism of the inhibitor was not obtained with any algal or non-algal sterol.

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A PHYSIOLOGICAL EXAMINATION AND TAXONOMIC REVISION OF SPECIES OF CHLORELLA ISOLATED FROM MARINE HABITATS

by

Rudolph E. Gross

Thesis submitted to the Faculty of the Graduate School
of the University of Maryland in partial fulfillment
of the requirements for the degree of
Master of Science
1964

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Introduction

Biologists have long recognized the properties of the genus Chlorella that make it an excellent tool for physiological research. However, widespread use of various isolates of Chlorella has made obvious the inadequacy of traditional taxonomic nomenclature for this genus when it is based mainly on morphology. Not enough visible characteristics are available in the simple spherical cells to differentiate between species, varieties, and strains of this genus. The literature is filled with data from experiments which cannot be repeated because of insufficient identification of the test organism. Therefore, it has become necessary to establish a classification of the natural species of Chlorella which will serve to identify species and to provide data concerning the unique physiological and biochemical characteristics of the members of this genus.

Departing from the descriptive approach, Shihira and Krauss (16) used the responses of 42 isolates to different media under controlled conditions as criteria for classification. Characteristics of species and strains were established during autotrophic, heterotrophic, and auxenotrophic growth using inorganic media with different nitrogen sources and supplements of carbohydrates, yeast extract, and vitamins.

Kessler and Soeder (8) have attempted to subdivide the genus into three large groups by determining hydrogenase activity, the

degree of de-coloration of nitrogen-starved cells during storage, and the responses of cells to ruthenium-red stain.

The majority of the species studied previously were isolated from fresh-water locations and for this reason the classification was necessarily restricted to fresh-water organisms. Although it has been known that Chlorella does occur in coastal waters, few comparative studies with salt-water and fresh-water isolates have been undertaken. Wetherell (19) grew fresh-water isolates of Chlorella in enriched natural sea water and reported that Chlorella pyrenoidosa Emerson's strain grows well in various concentrations of sea water, but that Sorokin's strain 7-11-05 did not. A morphological study of oceanic isolates was undertaken by Butcher (3) in order to identify those belonging to the genus Chlorella. George (6) has certain reservations as to the validity of Butcher's classification. George grew several of Butcher's marine strains and some fresh-water species in Föyn's Erdshreiber salt-water medium and found that the marine isolates were euryhaline. Fresh-water species, however, grew only in fresh water and in water of low salt concentrations. Recently Nakamura (12) reported an isolate of Chlorella from Ago Bay, Japan, which he tentatively called Chlorella pyrenoidosa type halophyla, suggesting preference for salt water. Harvey (7) made the following comments on salt-water Chlorella: "It is odd that 'marine species of Chlorella' are of rare occurrence in the open sea, in spite of their being not only unexacting, but also of their not being digested by copepods".

In the present study, the methods of Shihira and Krauss were extended to 30 isolates of Chlorella obtained from marine habitats.

The purpose of the investigation was to determine pertinent physiological characteristics of the marine species and thereby to broaden understanding of the genus. The research involved three phases: first, a survey of widely separated areas of sea and brackish waters to examine the distribution of the members of the genus Chlorella, and to isolate clonal cultures belonging to this genus; second, the identification of these isolates by physiological and morphological methods; and third, the determination of the degree to which these isolates are truly marine, as well as the degree to which selected fresh-water isolates can adapt to the marine environment.

Materials and Methods

Isolation of organisms:

One hundred samples of marine waters of approximately 250 ml each were collected between April 9 and September 26, 1962. The coastal waters of the eastern United States from Connecticut to Florida and Puerto Rico and the harbours of San Diego, California, and Acapulco, Mexico were sampled. Most samples were taken near the shore. Special emphasis was placed on the Chesapeake Bay and the waters of the Atlantic off the coast of Maryland. Here samples were taken at the shore as well as several miles off shore. All samples were transported in clean polyethylene bottles which had been filled to no more than 2/3 of their capacity in order to ensure an adequate oxygen supply. The water temperatures of the samples were not precisely controlled during transport, but efforts were made to avoid heating.

The salinity of the waters ranged from 2,000 ppm in some of the estuarine tributaries to full strength sea water, about 36,000 ppm in the coastal areas of the ocean. The salinities of the Chesapeake Bay samples ranged from 2,000 to 20,000 ppm.

Since species of Chlorella with different nutritional requirements were anticipated, a variety of media were employed. Aliquots of each water sample were introduced into a variety of media in order to give every opportunity for growth of fastidious species. Both natural and

artificial sea water media enriched with inorganic nutrients were used in several concentrations ranging from fresh-water to full strength sea water (Table I and II). A mixture of 11 vitamins (Table III) and yeast extract to get a concentration of 0.01% was added to all media. Media containing 0.01% glucose and 0.01% proteose-peptone were employed to stimulate growth of possible heterotrophs. As a basal medium for fresh-water growth the medium described by Shihira and Krauss (16) (Table IV) was adopted since it gives two nitrogen sources and a pH of 7.8 which is approximately that of sea-water samples from which algae were isolated. In certain media, the NH_4NO_3 was replaced by casein hydrolysate or KNO_3 as a nitrogen source. In such media, the buffer, which was 1.0 g K_2HPO_4 in the basal medium, was changed to 0.5 g K_2HPO_4 and 0.5 g KH_2PO_4. The resulting pH was 6.5. The six micronutrient elements, chelated as inner complex salts of ethylene-diaminetetraacetic acid after Thomas and Krauss (18), plus boric acid and molybdic anhydride were added to all media.

To facilitate the concentration of algal cells prior to isolation, a millipore filtre with a pore diameter of 3μ and filter disc diameter of 47 mm was employed. Algal cells were removed from the water samples by the filter. Large numbers of bacteria passed through and were eliminated. After filtration, the filters were placed on agar plates and incubated. In some cases smaller aliquots of the samples were placed directly on agar plates or were transferred into liquid media from which streaks were made on agar after green color became visible in the tubes. Incubation during isolation lasted from 5-30 days. All samples were incubated at an illuminance of 300 foot-

Table I

Enriched Natural Sea-water Medium

for Culture of Chlorella

Salt	Grams per liter of sea water **
Major Nutrients	
KNO_3	1.0
KH_2PO_4	0.5
K_2HPO_4	0.5
$MgSO_4 \cdot 7H_2O$	0.25
Micronutrients	
$Na_2 \cdot Mn \cdot EDTA$*	0.0071
$Na_2 \cdot Ca \cdot EDTA$	0.0071
$Na_2 \cdot Co \cdot EDTA$	0.0077
$Na_2 \cdot Cu \cdot EDTA$	0.0093
$Na_2 \cdot Zn \cdot EDTA$	0.0067
$Na_2 \cdot Fe \cdot EDTA$	0.038
MoO_3	0.001
H_3BO_4	0.010

* EDTA = Ethylenediaminetetraacetic acid

To prepare a solid medium add 15 g agar/l.

** The sea water was collected at the beach near Indian River Inlet, Md.

Table II

Enriched Artificial Sea-water Medium

for Culture of Chlorella*

Salt	Grams per liter of distilled water
Major Nutrients	
$NaCl$	24.72
KCl	0.68
$CaCl_2 \cdot 2H_2O$	1.40
$MgCl_2 \cdot 6H_2O$	4.78
$MgSO_4 \cdot 7H_2O$	6.30
$NaHCO_3$	0.097
$NaBr$	0.077
KNO_3	1.0
KH_2PO_4	0.5
Micronutrients	
$Na_2 \cdot Mn \cdot EDTA$**	0.0071
$Na_2 \cdot Ca \cdot EDTA$	0.0071
$Na_2 \cdot Co \cdot EDTA$	0.0077
$Na_2 \cdot Cu \cdot EDTA$	0.0093
$Na_2 \cdot Zn \cdot EDTA$	0.0067
$Na_2 \cdot Fe \cdot EDTA$	0.038
MoO_3	0.001
H_3BO_4	0.010

* Sea-water components according to the Marine Biological Laboratory, Woods Hole (4). Allen formula + sodium bromide, etc.

** EDTA = Ethylenediaminetetraacetic acid.
To prepare a solid medium add 15 g agar/l.

Table III

Vitamin Supplements for Culture Media

for Chlorella*

Vitamin	Milligrams per liter of medium
P-aminobenzoic acid	0.10
Biotin	0.005
Ca pantothenate	1.0
Choline chloride	5.0
O-phthalic acid	0.025
I-inositol	5.0
Nicotinic acid	1.0
Pyridoxin HCl	0.4
Riboflavin	0.02
Thiamin HCl	2.0
Vitamin B_{12}	0.001

* From Wetherell and Krauss (20).

Table IV

Fresh-water Medium for Culture

of Chlorella

Salt	Grams per liter of distilled water
Major Nutrients	
NH_4NO_3	1.00
K_2HPO_4	1.00
$MgSO_4 \cdot 7H_2O$	0.25
Micronutrients	
$Na_2 \cdot Mn \cdot EDTA$*	0.0071
$Na_2 \cdot Ca \cdot EDTA$	0.0071
$Na_2 \cdot Co \cdot EDTA$	0.0077
$Na_2 \cdot Cu \cdot EDTA$	0.0093
$Na_2 \cdot Zn \cdot EDTA$	0.0067
$Na_2 \cdot Fe \cdot EDTA$	0.038
MoO_3	0.001
H_3BO_4	0.010

* EDTA = Ethylenediaminetetraacetic acid.
To prepare a solid medium add 15 g agar/l.

candles from fluorescent light at 25°C. Every colony containing unicellular algae was picked off the plate by means of a micro-pipette and placed on an agar slant that contained the same ingredients as the previous medium. Seventy-five isolates of different unicellular green algae were collected, 50 of which were finally retained as superficially resembling Chlorella.

Purification of unialgal isolates:

Elimination of profuse bacterial contaminants was achieved by the application of standard bacteriological methods. A succession of streak plates eliminated most of the contaminants from the cultures. Using sterile techniques clonal colonies of alga were picked off the plates with micropipettes. Certain stubborn contaminants, having growth requirements similar to those of the algae, were so closely associated with them that streak plates were of no avail for purification. A mixture of 77 un/ml of penicillin and 25 µg/ml chloramphenicol (14) added to the agar successfully held down bacterial growth and by differential action permitted good algal growth.

Two cell washing techniques for the reduction of contaminants were tested. In one method, cells were drawn from a drop of liquid culture, which had been placed in a flat glass dish situated under a dissecting microscope, and released into another dish with sterile medium. This was repeated as often as judged necessary to lose the contaminant. A second washing method, described by McDaniel (11), used a 1% solution of phenol and a drop of surfactant. A large number of cells are washed at the same time. The phenol was supposed to kill

most bacteria but not the green algae. Centrifugation in distilled water acted as a rinse. Later the precipitate was streaked on agar plates. Both washing methods were rarely successful when applied to these isolates.

After the initial purification, each algal isolate was checked in a rich organic medium containing 0.1% trypticase soy broth, 0.1% glucose, and 0.01% yeast extract in addition to the basal inorganic salts. If no cloudiness had developed after three days of incubation in the light at room temperature followed by three days of incubation in the dark at 37°C, the culture was judged free of obvious contaminants.

Physiological examination of the isolates:

The work in this section was based on the physiological approach developed by Shihira and Krauss (16). The basal experimental medium is shown in Table IV. Stock cultures are kept on agar slants of Bristol's medium. The heterotrophic capabilities of the isolates were tested by adding the respective sugars to basal medium to obtain a concentration of 0.1%. The growth response over that on basal medium was measured in both light and darkness. The growth response to 0.1% acetate was also measured. To check for inhibitory action, 1.0% acetate in basal medium was employed for cultures in the light. Possible growth factor requirements were sought through use of 0.01% yeast extract (DIFCO-Certified Bacto Yeast Extract). Previous studies have shown thiamin to be the vitamin required by some species of Chlorella. Isolates were therefore tested by their response to 10 µg of thiamin per liter of medium. The preference or requirement

for a specific nitrogen source was established using either 0.2 g KNO_3 per liter, 0.1 g NH_4Cl per liter or 0.1% casein hydrolysate (DIFCO vitamin-free casamino acids) instead of NH_4NO_3 as the nitrogen source. When KNO_3 was used, the buffer system was changed to 0.5 g/l each of monobasic and dibasic potassium phosphate.

Since high temperature may cause side reactions that alter the composition of chemical solutions, all media were sterilized by filtration with millipore filter discs having a pore size of 0.45μ (HA) to assure uniform composition from test to test. Ten ml of medium were dispensed aseptically through a Cornwall continuous pipettor into steam sterilized test tubes 15 cm long and 18 mm wide. Each tube was plugged with cotton.

Duplicate tubes were used in all tests, and each test was repeated at least once. To avoid carryover of nutrients from slants, and to assure uniformity, all isolates were grown on basal liquid medium before inoculation into test media. When preference for a nutrient was observed, the inoculum was taken from the preferred medium for the repeat test. The standard optical density for any source of inoculum was 0.5 as measured in 18 mm test tubes on a Bausch and Lomb spectrophotometer at 550 mμ. Using sterile technique 0.1 ml of the source-culture was added to 10 ml of the test medium. This represents a 100 fold dilution and gives a starting O.D. of 0.005. In the cases of certain slow growing cultures, the source O.D. was 0.25 and a double amount of the source-culture was used for inoculation. Growth was measured in terms of O.D. after three days and again after 5 days. If results were negative, the tubes were

incubated for two more days. If the reading was higher than 0.2 units of O.D. after three days, the test was terminated.

During the incubation period all tubes were slanted at 23° from the horizontal in racks on an Eberbach reciprocating shaker. The shaker had an amplitude of 3 inches at the rate of 68 cycles/minute which was maintained throughout incubation. Fluorescent lamps over the shaker provided 400 foot-candles of illuminance. For studies of growth in darkness the tubes were placed on the same shaker, but wrapped in aluminum foil with allowance for ample gas exchange. The temperature in the room was held at 24°C.

The high-temperature isolates were grown on medium employing 0.02% KNO_3 as the nitrogen source. Before exposing the organisms to saturating light and high temperature, several serial transfers were incubated at reduced light and at 38°C in test tubes through which a 1.0% CO_2-in-air mixture was bubbled.

Microphotography:

Photomicrographs were taken with a Kodak camera body attached to a Bausch and Lomb Dynazoom Laboratory Microscope equipped with an objective apochromat 90X oil immersion lens, N.A. 1.3. The temperature of the light used was about 3400°K. The photographs were taken at 1/25 second with the iris diaphragm 3/4 closed to obtain maximum permissible contrast. Before the light entered the substage condenser, it was filtered with a Didymium BG 20 filter. Kodachrome II Professional Type A film for photoflood (KRA 135) gave 2" x 2" transparencies which were 270 times the enlargements of the cells.

Results and Discussion

Experimental Results:

When the isolation procedures were completed, a total of 50 unialgal clones of small, spherical to oval, green, unicellular organisms were available. The most successful isolation technique had proved to be that of filtering the samples through a millipore filter and subsequently blotting the filter discs on agar plates. Because most samples contained algae of predominantly one species, it was possible to make several blottings onto successive agar plates and to obtain similar numbers of colonies of the same species from each transfer. Such serial blotting, alternatively on fresh-water and salt-water agar plates, demonstrated early in the study that all the isolates would grow on fresh-water agar, but many failed to grow on salt-water agar.

Of the 50 unialgal isolates, 49 were freed of all contaminants. Agar streak plates with 77 un/ml penicillin and 25 mg/ml chloramphenicol appeared to be the most effective in eliminating bacteria and fungi. Gram negative rods, and, in some instances, bacteria which liquified agar, were the most difficult to destroy selectively.

During repeated observations of the individual cells in many kinds of media, differences in morphology were observed and recorded. Seven isolates were eliminated from the study because they were clearly not members of the genus Chlorella. Fifteen isolates, the descriptions of which are included in the appendix, were more closely related to

Chlorella. These organisms, however, differed morphologically from Chlorella by such characteristics as slight unipolar thickenings of the cell wall, string-like attachments of released daughter cells to the mother cell wall, or minute setae and spines. One isolate reproduced by aplanospores and at the same time some cells elongated and divided by fission. Two of the isolates produced cells which were indistinguishable from Chlorella, but many atypical cells also appeared in cultures of these two organisms. Most of the Chlorella-like organisms were identified as representatives of the genera Lagerheimia, Westella, and Oocystis.

Twenty-nine isolates, identified by morphology as Chlorella, were given a series of physiological tests following those developed by Shihira and Krauss (1964). Also included were four organisms received from Dr. Butcher. Relationships were apparent between isolates from widely separated geographical areas, which also differed in salinity. Table V lists the species that were identified or established in this study. In several cases a number of isolates proved to be representatives of the same species. It can be seen even from this limited sampling that the species found in marine habitats are cosmopolitan.

The responses of the species to selected carbon sources in the light and darkness are given in Table VI. C. autotrophica var. atypica, C. verautotrophica and C. verautotrophica var. minuta are not supported by any hexose tested. This suggests a strictly autotrophic metabolism for these organisms. Some organisms, such as C. regularis var. minima and C. emersonii var. globosa, metabolize four hexoses, while others are more selective in their heterotrophic growth and use only selected

Table V

Species and Sub-species of *Chlorella* Isolated from Marine Habitats Primarily Off the Atlantic and Pacific Coasts of North America in 1962

Organism	Culture Collection No.*	Month of Collection	Location	Salinity at Point of Collection
C. nocturna	MCC 3	June	Mouth of Mystic River, Conn.	Tidal waters
C. regularis var. *umbricata*	MCC 4 a	July	2 mi. off Bethany, Md.	ca. 36,000 ppm
	4 b	June	Bay Ridge, Chesapeake Bay, Md.	ca. 10,000 ppm
	4 c	June	Potomac River, off Cobb Island, Md.	ca. 8,000 ppm
	4 d	May	Wicomico River, Chaptico Bay, Md.	ca. 7,000 ppm
	4 e	May	Patuxent River, at Benedict Md.	ca. 4,000 ppm
	4 f	April	Severn River (3 mi. from mouth), Md.	ca. 2,000 ppm
C. regularis var. *minima*	MCC 5	July	5 mi. east of Point Lookout, Chesapeake Bay, Md.	ca. 1,600 ppm
C. infusionum var. *acetophila*	MCC 6 a	July	Off Turkey Point, Chesapeake Bay, Md.	ca. 1,000 ppm
	6 b	July	Chesapeake and Delaware Canal, Delaware	Unknown

Table V continued

Organism	Culture Collection No.*	Month of Collection	Location	Salinity at Point of Collection
C. acuminata var. pompanoensis	MCC 7 a 7 b	July	Pompano Beach, Florida Off Aberdeen, Chesapeake Bay, Md.	ca. 36,000 ppm ca. 5,000 ppm
C. sorokiniana var. atlanticensis	MCC 8	September	San Juan harbour, Puerto Rico	Tidal waters
C. sorokiniana var. pacificensis	MCC 9	September	Acapulco harbour, Mexico	Tidal waters
C. autotrophica var. atypica	MCC 10		(Isolated by Dr. R. Lewin)	
C. emersonii var. globosa	MCC 11	May	Wicomico River, Chaptico Bay, Md.	ca. 7,000 ppm
C. anitrata	MCC 12	July	Canal Pompano to W. Hollywood, Florida	Tidal waters
C. anitrata var. minor	MCC 13	July	Intracoastal Waterway, Dania, Florida	Tidal waters
C. glucotropha	MCC 14	July	Salt Peter Creek, SE of Chase, Md.	ca. 4,000 ppm

Table V continued

Organism	Culture Collection No.*	Month of Collection	Location	Salinity at Point of Collection
C. glucotropha var. *caribbeanensis*	MCC 15	September	San Juan harbour, Puerto Rico	Tidal waters
C. verautotrophica	MCC 16 a	July	Pompano Beach, Florida	ca. 36,000 ppm
	16 b	July	East of Annapolis, Chesapeake Bay, Md.	ca. 15,000 ppm
C. verautotrophica var. *minuta*	MCC 17	July	Canal Pompano to W. Hollywood, Florida	Tidal waters
C. pyrenoidosa	MCC 18 a	July	2 mi. off Ocean City, Md.	ca. 36,000 ppm
	18 b	July	Canal Pompano to W. Hollywood, Florida	Tidal waters
C. parva	MCC 19 a	July	Delaware Bay (near mouth), Delaware	ca. 25,000 ppm
	19 b	July	Delaware Bay (center), Delaware	ca. 15,000 ppm
C. gravata	MCC 20 a	July	Off Rock Hall Landing, Chesapeake Bay, Md.	ca. 12,000 ppm
	20 b	July	Chesapeake Bay near Annapolis, Md.	ca. 14,000 ppm
C. salina	PL 86		Oyster breeding tanks, Conway, N. Wales, England	
	CRA-1		Crouch estuary, Essex, England	

Table V continued

Organism	Culture Collection No.*	Month of Collection	Location	Salinity at Point of Collection
C. sparkii	CV 94		Oyster breeding tanks Conway, N. Wales, England	
	PE 1		Port Erin, England	

* MCC = Maryland Culture Collection, Department of Botany, University of Maryland, College Park, Maryland
PL, CRA, CV, PE = Code numbers, R. W. Butcher, Marine Fisheries Laboratory, BURNHAM-ON-CROUCH, Essex, England.

Table VI

The Growth of Marine Isolates of *Chlorella* in Light and Darkness on Inorganic Media Supplemented with One Gram of Carbohydrate per Liter

Organism	Control O.D. X 10	Glucose		Galactose		Fructose		Mannose		Lactose		Sucrose		Maltose		Raffinose		Dextrin	
		L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D
C. nocturna	3	0	+	0	S	0	0	-	0	0	0	0	0	0	0	0	0	0	0
C. regularis var. *umbricata*	2	1	++	2	+	2	++	-	0	0	S	0	0	0	0	0	0	0	0
C. regularis var. *minima*	4	6	+	6	S	4	0	6	+	0	0	0	0	0	0	0	0	0	0
C. infusionum var. *acetophila*	3	5	++	0	0	0	0	-	0	0	0	0	0	0	0	0	0	0	0
C. acuminata var. *pompanoensis*	4	1	+	1	S	0	0	0	+	0	0	0	0	0	0	0	0	0	0
C. sorokiniana var. *atlanticensis*	3	8	+	4	S	0	0	-	0	0	0	0	0	0	0	0	0	0	0
C. sorokiniana var. *pacificensis*	5	8	++	4	S	0	0	-	0	0	0	0	0	0	0	0	0	0	0
C. autotrophica var. *atypica*	2.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. emersonii var. *globosa*	3	4	+	0	S	4	+	4	+	0	0	0	0	0	0	0	0	0	0
C. anitrata	3	2	+	0	S	0	0	2	S	0	0	0	0	0	0	0	0	0	0
C. anitrata var. *minor*	3	3	+	0	S	0	0	1	S	0	0	0	0	0	0	0	0	0	0
C. glucotropha	4	0	+	0	0	0	0	-	0	0	0	0	0	0	0	0	0	0	0
C. glucotropha var. *caribbeanensis*	3	1	S	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table VI continued

Organism	Control O.D. X 10	Glucose		Galactose		Fructose		Mannose		Lactose		Sucrose		Maltose		Raffinose		Dextrin	
		L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D
C. verautotrophica	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. verautotrophica var. *minuta*	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. pyrenoidosa	3	3	+	1	S	0	0	1	S	0	0	0	0	0	0	0	0	0	0
C. parva	5	8	+	8	S	2	0	-	0	0	0	0	0	0	0	0	0	0	0
C. salina	2	4	S	3	S	1	0	-	0	0	0	0	0	0	0	0	0	0	0

0 = no stimulation over basal medium in light.
1-8 = stimulation over growth on basal medium in light in terms of tenths of an O.D. unit (e.g., 3 = O.D. in basal medium + .3).
S = very slight response.
(-) = inhibition of growth in basal medium and light.
(+) = support of growth in the dark of at least 0.2 O.D. units above control.
(++) = support of growth in the dark almost equivalent to growth on glucose in the light.

sugars either in light or darkness or both. The degree of heterotrophy in an organism probably cannot be judged from the number of organic carbon sources it can use, or the growth rate it will attain on any particular source. C. regularis var. minima grows very well on glucose, galactose, and fructose in the light and its growth rate is equally good on mannose; C. parva has a very similar metabolic pattern with respect to the first three sugars, but mannose does not support growth, in fact it is inhibitory. Some species such as C. parva bleach when grown heterotrophically. Others such as C. infusionum var. acetophila keep a deep green color. In general it was observed that organisms showing little or no response to any sugar under any circumstances are normally slow growing in inorganic media and demonstrate a high degree of inhibition by acetate. Isolates stimulated by galactose and/or fructose in light or darkness are also stimulated by glucose, but not vice versa. Moreover if growth on galactose and fructose is good it will also be good on glucose. C. regularis var. umbricata, as isolated and tested separately from six locations, showed some growth on lactose in darkness. It constitutes the only case in this particular study where an organism responded to a disaccharide.

None of the organisms grew on sucrose, maltose, raffinose or dextrin. It should be kept in mind that testing was of a short-term nature and growth might have occurred after prolonged incubation. Two isolates of the genus Westella from waters from Florida and Maryland were established as the same species on the basis of their extremely slow growth on sucrose after six weeks of standing in darkness.

It is possible that autoclaving might have helped to initiate the breakdown of the polysaccharides so that growth might occur.

Table VII shows the effects of two concentrations of sodium acetate supplement. C. autotrophica var. atypica which did not grow on any of the carbohydrates used, showed slight growth on acetate in darkness. It should be noted that inhibition by 1% acetate in the light was not correlated with the capability to metabolize 0.1% acetate. C. glucotropha was stimulated by 0.1% acetate in the light and darkness, but was completely inhibited by 1% acetate in the light. Other species followed the same pattern to various degrees. C. nocturna is the only species listed that showed good acceleration on acetate in the light, but none on glucose in the light. All isolates strongly inhibited by mannose also showed reduced growth on 1% acetate, but some organisms stimulated by mannose were also inhibited by the acetate.

The results of tests using four different nitrogen sources are shown in Table VIII. Most isolates could use all nitrogen sources equally well, and differences in synthetic capability with respect to a nitrogen source under the conditions of the tests were not very striking. Several exceptions to this generalization exist. C. salina showed greatly reduced growth on NH_3 which is a characteristic not previously reported for any species of this genus. If given NH_4NO_3 the growth of this organism is a little greater than on KNO_3 alone. C. pyrenoidosa did not show any appreciable growth when KNO_3 was the N source; NH_4NO_3, NH_4Cl, and casein hydrolysate supported growth equally well. A similar situation although less clear-cut, exists

Table VII

The Growth of Marine Isolates of *Chlorella* in Light and Darkness On Inorganic Media Supplemented with Sodium Acetate

Organism	Terminal O.D. of Control	0.1% Acetate*		1% Acetate**
		Light	Dark	Light
C. nocturna	3	4	3	+
C. regularis var. *umbricata*	2	2	3	-
C. regularis var. *minima*	4	0	S	0
C. infusionum var. *acetophila*	3	4	2	-
C. acuminata var. *pompanoensis*	4	2	S	0
C. sorokiniana var. *atlanticensis*	3	4	2	-
C. sorokiniana var. *pacificensis*	5	5	3	-
C. autotrophica var. *atypica*	2.5	0	S	0
C. emersonii var. *globosa*	3	0	0	+
C. anitrata var. *minor*	3	3	2	-
C. anitrata	3	2	1	-
C. glucotropha	4	2	2	0
C. glucotropha var. *caribbeanensis*	3	0	0	0

Table VII continued

Organism	Terminal O.D. of Control	0.1% Acetate* Light	0.1% Acetate* Dark	1% Acetate** Light
C. verautotrophica	4	0	0	0
C. verautotrophica var. minuta	2	0	0	-
C. pyrenoidosa	3	4	S	+
C. parva	5	0	2	-
C. salina	2	2	S	-

* Numbers represent stimulation over inorganic medium without an acetate supplement in terms of tenth of an O.D. unit (e.g., 3 = O.D. in basal medium + .3).

**0 = strong inhibition, almost equivalent to no growth.
(-) = reduced growth.
(+) = growth at least equal to inorganic medium.

Table VIII

Growth of Marine Isolates of *Chlorella* in the Light on Media Supplying Nitrogen from Different Sources*

Organism	Casein Hydrolysate	NH_4Cl	KNO_3	NH_4NO_3
C. nocturna	7	7	(-)7	7
C. regularis var. *umbricata*	7	7	(+)7	7
C. regularis var. *minima*	7	7	(+)7	7
C. infusionum var. *acetophila*	7	7	(+)7	7
C. acuminata var. *pompanoensis*	6	7	7	7
C. sorokiniana var. *atlanticensis*	8	(+)7	(+)7	7
C. sorokiniana var. *pacificensis*	8	8	8	8
C. autotrophica var. *atypica*	5	5	5	5
C. emersonii var. *globosa*	7	7	7	7
C. anitrata	6	6	0	0
C. anitrata var. *minor*	6	6	0	0
C. glucotropha	6	7	6	6
C. glucotropha var. *caribbeanensis*	(+)7	(+)7	(+)7	7
C. verautotrophica	6	7	6	(+)6

Table VIII continued

Organism	Casein Hydrolysate	NH_4Cl	KNO_3	NH_4NO_3
C. verautotrophica var. *minuta*	3	5	(+)0	3
C. pyrenoidosa	7	7	(+)0	7
C. parva	(+)7	(+)7	8	8
C. salina	5	1	5	6

* Figures represent the numbers of doublings for a five day period.

in the case of C. verautotrophica var. minuta. The growth of this species was much reduced on KNO_3, and higher on the other three N sources, but growth generally was poor and rates were fluctuating. C. anitrata and C. anitrata var. minor were not supported by nitrate. Growth was equally good on NH_4Cl or casein hydrolysate. In contrast to the other species not supported by KNO_3, C. anitrata and C. anitrata var. minor did not grow when NH_4NO_3 was used as a N source. The cells attained diameters which were four times that of normal cells in media where NH_3 was the sole N source, but no release of daughter cells took place and eventually the giant cells disintegrated. Under the conditions of these tests nitrate seemed to have an inhibitory effect.

Not obvious from the data of Table VIII is the effect of different N sources on the morphology of the cells. C. emersonii var. globosa was supported equally well by all N sources used, and growth rates were identical as measured in terms of optical density in our tests. However, the chromatophore was mantle-shaped and parietal when grown on KNO_3 but changed to net-shaped and parietal when NH_4Cl was used as a nitrogen source. Casein hydrolysate caused the cells to grow a little larger, and the chromatophore morphology appeared to be a mixture of the two previous types.

Sea-water Tests:

Organisms isolated from saline surroundings can be designated marine species or salt-water species for two reasons. First, because of the apparent natural habitat of the organism, and second, because they behave differently from known fresh-water isolates of the same

genus when put into sea-water media under artificial culture conditions. In order to determine whether the present isolates can satisfy the second criterion a group of known fresh-water isolates, some of them the same species as the marine isolates, were tested in sea-water media of different concentrations for comparison with the marine isolates grown under similar conditions. The following species were used for this experiment:

Chlorella ellipsoidea Gerneck

Chlorella vulgaris Beyerinck

Chlorella sorokiniana Shihira and Krauss

Chlorella vannielii Shihira and Krauss

Chlorella autotrophica Shihira and Krauss

Chlorella nocturna Shihira and Krauss

Natural sea water enriched with inorganic nutrients was used at three concentrations--1/4, 1/2, and full-strength. The same medium supplemented with 0.1% glucose was also employed in the darkness at concentrations of 1/2 and full-strength.

The inoculum for these tests was taken from basal, liquid medium at a nutrient concentration twice that normally used in order to partially overcome the shock to the cells by the change of osmotic conditions. The initial transfer from the fresh-water medium was made into the 1/4 sea-water concentration. After growth had occurred subsequent transfers were made, in a similar fashion, to media containing one-half and full-strength sea-water.

The results of the sea-water tests without organic supplement, Table IX, showed that the growth-rates of all species reduced as

the salinity was increased. The salt-water isolates, in general, did not grow much better in the saline media than the fresh-water organisms. Both high temperature isolates, C. sorokiniana var. atlanticensis and var. pacificensis, grew better in saline media than the other isolates; C. sorokiniana, also a high temperature organism, grew best among the fresh-water isolates, but at high salt concentrations it did not achieve the growth rates of the high temperature varieties isolated from sea-water.

Organisms growing on saline glucose media in the darkness, Table X, do not grow as rapidly as on inorganic saline media in the light. This suggests the photosynthesis-linked ion-pump in Ulva demonstrated by Scott and Hayward (15).

It is of interest to observe a series of isolates such as those of C. regularis var. umbricata which came from six different locations varying in salinity from 2,000 ppm to 36,000 ppm. In all salt-water tests these isolates showed no differences as to physiology or morphology and all stopped growing at a relatively low salt concentration. This agrees with the general observation that the rate of growth displayed by different isolates in sea-water is not at all related to the salinity of their apparent natural habitat. Some isolates, originating from full-strength sea-water, grew only in media that were 50% of the osmotic pressure at the sampling point. Whether differences in osmotic pressure, pH, ionic ratios, organic substances, temperature, light, dissolved gases, vitamins or other factors were the cause of this apparent discrepancy was not determined in this study.

Table IX

The Effects of Salt-water on Marine Species

And Certain Fresh-water Species of *Chlorella*[*]

Organisms	Fresh-water Medium[**]	1/4 Natural Sea-water[**]	1/2 Natural Sea-water[***]	Full Strength Natural Sea-water[****]
Marine Isolates:				
C. nocturna	3	3	1	0
C. regularis var. *umbricata*	2	2	1	0
C. regularis var. *minima*	4	1	L	0
C. infusionum var. *acetophila*	3	3	1	0
C. acuminata var. *pompanoensis*	4	1	L	0
C. sorokiniana var. *atlanticensis*	4	5	7	3
C. sorokiniana var. *pacificensis*	5	5	6	3
C. autotrophica var. *atypica*	2.5	1	2	L

Table IX continued

Organism	Fresh-water Medium**	1/4 Natural Sea-water**	1/2 Natural Sea-water***	Full Strength Natural Sea-water****
C. emersonii var. globosa	3	2	1	0
C. anitrata	3	L	0	0
C. anitrata var. minor	3	L	0	0
C. glucotropha	4	3	1	0
C. glucotropha var. caribbeanensis	3	L	L	0
C. verautotrophica	4	2	1	0
C. verautotrophica var. minuta	2	L	0	0
C. pyrenoidosa	3	L	0	0
C. parva	5	4	1	0
C. gravata	1	L	L	0
C. salina	2	3	2	1 (bleached)
Fresh-water Isolates:				
C. vulgaris	3	1	L	0

Table IX continued

Organism	Fresh-water Medium**	1/4 Natural Sea-water**	1/2 Natural Sea-water***	Full Strength Natural Sea-water****
C. sorokiniana	4	2	3	L
C. pyrenoidosa	3	2	1	0
C. autotrophica	3	4	3	0
C. nocturna	3	2	1	0
C. ellipsoidea	4	3	1	0

* The numbers given represent terminal growth in tenths of optical density units using 18 mm test-tubes in a B&L spectrophotometer at 550 mμ. (L) represents growth less than 0.1 O.D. unit during a particular period. (0) represents no growth of any significance.
** Three days of incubation.
*** Five days of incubation.
**** One week of incubation.

Table X

Growth in Darkness of Marine and Fresh-water Isolates of *Chlorella* on a Natural Sea-water Medium Supplemented with 0.1% Glucose*

Organism	Fresh-water Control**	1/2 Natural Sea-water***	Full Strength Natural Sea-water***
Marine Isolates:			
C. regularis var. *umbricata*	3	L	0
C. emersonii var. *globosa*	5	L	0
C. sorokiniana var. *atlanticensis*	5	4	L
C. sorokiniana var. *pacificensis*	10	2	L
C. salina	1	1	0
Fresh-water Isolates:			
C. ellipsoidea	5	1	0
C. sorokiniana	8	2	0

* The numbers given represent terminal growth in tenths of optical density units using 18 mm test-tubes in a B&L spectrophotometer at 550 mμ. (L) represents growth less than 0.1 O.D. unit during a particular period. (0) represents no growth of any significance.
** Terminal growth after three days of incubation.
*** Terminal growth after seven days of incubation.

In order to explore the possibility that some isolates did grow, but very slowly, in sea-water they were left standing under reduced light for six weeks after the normal test period. However, no significant amount of growth was observed. On the contrary, cells of the inoculum seemed to bleach faster than in fresh-water. Such bleached cells were still viable in many cases and normal growth could be achieved by transferring them into new media with an osmotic pressure closer to that of fresh-water.

From the tests it seems that Chlorella cannot be separated into fresh-water and salt-water species. The same species can be found in nature in both types of water. However, it would not be accurate to refer to the genus as euryhaline as all species show a reduction in growth rate when the concentration of the medium reaches 10,000 ppm. This goes far toward explaining why Chlorella is not a dominant marine genus. All species in diluted sea-water in the light produce cells of at least twice the size normally found in fresh-water. The enlarged cells also demonstrate a tendency toward more granular chloroplasts. Of the marine isolates C. sorokiniana var. atlanticensis appears to be the species most adapted to growth in saline habitats. Nevertheless it exhibited the same symptoms of salt toxicity as the other species.

Possibly, the cells found in brackish and sea-water were very slowly and gradually washed into them from fresh-water; slow enough to allow the cells an adequate adjustment period. It is obvious that the species found by the 100 ml aliquot sampling technique must have been common and viable in each. It was possible to isolate a species

of Chlorella or closely related unicellular green algae, from at least half the water samples. The production of clonal mutants in the absence of sexual recombination, or an inherent capacity to adapt to a changing environment may allow the species of Chlorella to exist either as fresh-water organisms or as inhabitants of a marine environment.

Description of Species:

Complete descriptions of all the species isolated from marine habitats are given even though some have been described earlier in the monograph by Shihira and Krauss (16). In certain cases minor amendments to the description have been made and the reasons for the changes are discussed. At the conclusion of the description of the species is a physiological key to the known marine species of Chlorella.

Chlorella nocturna Shihira and Krauss 1964:

Figures (1,2)

Cells always spherical, 4-8μ in diameter, nearer to 10μ when grown on glucose. Chromatophores in small cells shallow, cup-shaped, large cells filled with a slightly granular chromatophore; green but turning white in old cultures in light; deep green in darkness when supplied glucose. Pyrenoid present.

Good growth on inorganic liquid media in light. Glucose not accelerative, rather inhibitory in light; glucose stimulating in darkness. No other sugars in light stimulate, except some slight acceleration due to galactose. Mannose tends to be strongly inhibitory in light; only glucose is stimulating in darkness; acetate is stimulatory in the light.

Growth on all nitrogen sources is approximately the same. Yeast extract is not accelerative on glucose or inorganic media.

Culture number MCC 3 isolated by Gross, from the mouth of Mystic River, Connecticut.

The marine isolate is morphologically identical to C. nocturna. All tests with carbohydrate supplements show the same synthetic pattern for both the marine isolate and the species. In addition both are strongly inhibited by mannose. Both, the marine isolate and the type species, grow better on acetate than on glucose in the light. This is a rare characteristic in the genus.

Chlorella regularis (Artari) Oltmanns var. umbricata Shihira and Krauss 1964:

Figures (3,4)

Cells spherical, 4-8μ in diameter, nearer 10μ when grown on glucose. Chromatophore parietal, mantle-shaped; deep green in inorganic media, and very pale green and granular when grown on glucose media in darkness. Distinct pyrenoid present. Daughter cells upon release are typically irregularly shaped.

Good growth on inorganic liquid media. Glucose stimulates good growth in the dark, but has little effect on growth in the light. Galactose and fructose stimulate growth in light and darkness. Mannose slows growth in inorganic media in light and does not support growth in darkness. Lactose is ineffective in light, but supports some growth in darkness. Acetate is also stimulatory in darkness.

Ammonium, nitrate, and casein hydrolysate support growth, but

there is a slight preference for nitrate.

Yeast extract is not stimulatory in inorganic and glucose media.

Culture numbers MCC 4 a, 4 b, 4 c, 4 d, 4 e, and 4 f isolated by Gross from: a) 2 mi. off Bethany, Maryland (Atlantic), b) Bay Ridge, Chesapeake Bay, Maryland, c) Potomac River, Cobb Island, Maryland, d) Wicomico River, Chaptico Bay, Maryland, e) Patuxent River at Benedict, Maryland, f) 3 mi. from mouth of Severn River, Maryland.

The morphological descriptions of the marine isolates match those of the variety umbricata. In addition it was observed that the growth of the marine isolates was not enhanced by light when grown on glucose, and that they were stimulated by lactose in the dark. Both characteristics helped to establish the variety umbricata. The marine isolates match the physiological description of C. regularis var. umbricata in all the other major points and it was concluded that these organisms are identical.

Chlorella regularis var. minima var. nov.

Figures (5,6)

Cells spherical to slightly ovoid, 3-6μ in diameter, closer to 8μ when grown on glucose media. Chromatophore parietal mantle-shaped in inorganic media, green, changing to granular and pale green in glucose media. Pyrenoid always present.

Grows slowly on inorganic media. Glucose, galactose, mannose, and fructose equally accelerate growth in the light, and all except fructose support some growth in darkness; fructose is ineffective. Acetate supports very slight growth in the dark; 1% acetate is strongly

inhibitory in the light.

Nitrate, ammonia, and casein hydrolysate serve equally as N sources; at times a slight nitrate preference has been observed. Yeast extract is ineffective in inorganic or glucose media. No other species shows a close resemblance to the physiological pattern of this isolate.

This organism closely resembles Chlorella regularis Shihira and Krauss. It has the typical parietal chloroplast and is similar in its responses to nitrogen sources and acetate. However it is somewhat smaller in the light than the species and the varieties umbricata and atypica. Its most conspicuous physiological characteristic is the clear stimulation by mannose in both light and darkness.

Chlorella infusionum var. acetophila var. nov.

Figures (7,8)

Cells spherical to ovoid, 2-3μ in diameter, closer to 6μ for mother cells containing normally 32 daughter cells in inorganic media, and for cells grown on glucose in darkness. Chromatophore cup-shaped, not filling cells, deep green in inorganic medium becomes pale green on glucose in darkness.

Good growth in inorganic media. Glucose strongly stimulatory in light and darkness, galactose and fructose are ineffective; mannose strongly inhibits growth in light. Acetate stimulates well in both light and darkness.

Ammonia, nitrate, and casein hydrolysate serve equally well as N sources, however a slight preference for nitrate may exist.

Yeast extract is ineffective.

Type culture number MCC 6 a isolated by Gross from off Turkey Point, Chesapeake Bay, Maryland.

Identical strain MCC 6 b isolated from Chesapeake and Delaware Canal, Delaware.

The species and this variety cannot be distinguished morphologically. Both grow well on inorganic media and on glucose in the light and darkness. Mannose is equally inhibitory. The variety acetophila is strongly stimulated by acetate while the species is not, setting the variety apart from the species. It also differs from C. infusionum var. auxenophila which is stimulated by yeast extract, and which does grow more rapidly on acetate in the light but not in darkness.

Chlorella acuminata var. pompanoensis var. nov.

Figures (9,10)

Cells normally ovoid, 3-8μ in diameter, nearer to 10μ when grown on glucose media. Chromatophore mantle-shaped, becoming granular in sugar media; always green. Pyrenoid present.

Growth on inorganic media is slow; glucose gives some stimulation to growth in light and supports slow growth in darkness; galactose and mannose support very weak growth in the dark; fructose is ineffective. Acetate supports growth slightly in darkness; 1% acetate is strongly inhibitory in the light. Poor growth on any media in darkness.

Nitrate and ammonia serve equally well as N sources. Growth is somewhat reduced in media with casein hydrolysate as a N source.

Yeast extract added to inorganic or glucose media was ineffective.

Type culture number MCC 7 a isolated by Gross from Pompano Beach, Florida.

Identical strain MCC 7 b isolated from off Aberdeen, Chesapeake Bay, Maryland.

In inorganic medium the species and the variety have the same average dimensions, however, the species is ellipsoid while the variety is ovoid. When grown on glucose both become round and have the same increased diameter.

Both grow slowly on inorganic medium and are only slightly stimulated by glucose in the dark. Although the growth pattern is the same for both the species and the variety when grown on galactose and mannose in the dark, the response to fructose is different. The variety pompanoensis does not grow on fructose in darkness. Furthermore this variety grows weakly on acetate in darkness but the species does not. In general both the species and the variety pompanoensis exhibit poor growth on any medium in darkness.

Chlorella sorokiniana var. atlanticensis var. nov.

Figures (11,12)

Cells ovoid, 1-3μ in diameter closer to 3μ when grown on glucose. Chromatophore cup-shaped in young cells, becomes mantle-shaped in older cells; dark-grown cells stay green in liquid culture; growth on inorganic agar slants is thin and pale green, turning white quickly. Pyrenoid present.

Moderate growth on inorganic liquid medium. Glucose supports

very good growth in light and moderate growth in the dark. Galactose stimulates growth in light and weakly stimulates growth in darkness. Mannose only slightly inhibits growth in the light, and does not support any dark growth. Other sugars not effective. Acetate is weakly stimulatory in light and darkness.

Ammonia and nitrate utilized equally. Casein hydrolysate as a N source supports growth slightly better than NH_4NO_3.

When grown at 24°C and illuminated with 500 f-c light intensity the isolate reaches a growth rate of three doublings per day in 1% CO_2 enriched air bubble cultures. Raising the temperature to 38°C and saturating light intensity the number of doublings per day is in excess of six, indicating a high-temperature strain.

Type culture number MCC 8 isolated by Gross from San Juan harbour, Puerto Rico.

The diameter of the variety atlanticensis is slightly less than that of the species. Growth on inorganic medium is generally slower for the variety when compared with the species. The variety also grows more slowly on acetate in the light. Mannose inhibition is stronger for the species than it is for the variety atlanticensis. Growth on glucose in the light is equal for both, but growth is markedly reduced for the variety in darkness.

A strong resemblance is the increased growth shown by both the species and the variety, when grown at 38°C and at a high light intensity.

Chlorella sorokiniana var. pacificensis var. nov.

Figures (13,14)

Cells slightly ellipsoidal, 2-4μ in length, tend to become more

spherical and close to 5μ in diameter in glucose media. Chromatophore bowl-shaped and smooth in inorganic media; becomes more granular in glucose medium in darkness, but stays dark green.

Good growth on inorganic liquid medium. Glucose supports excellent growth in light and darkness. Galactose stimulates good growth in light and moderate growth in darkness. Mannose inhibits growth slightly, and acetate supports good growth in the light and some growth in darkness.

Ammonia, nitrate and casein hydrolysate are equally utilized as N sources.

In inorganic and glucose medium, the cells of this isolate exude a sticky substance causing the cells to clump; shaking of the tubes will separate these clumps. Stained slides reveal small amounts of a shiny layer trailing the clumps of cells; it does not have the appearance of a capsule.

The normal growth rate of this isolate in inorganic medium at 24°C and 500 f-c illumination is approximately 2.5 doublings per day when 1% CO_2-in-air is bubbled through the culture. This growth rate can be increased to over 5 doublings per day by raising the temperature to 38°C under saturating light conditions, indicating a high-temperature strain.

Type culture number MCC 9 isolated by Gross from Acapulco harbour, Mexico.

Morphologically the species, the variety pacificensis, and the variety atlanticensis are similar. Growth on different organic supplements also shows a resemblance. The variety pacificensis

differs from both by a somewhat slower growth rate when grown at high temperature, and also by a slimy substance formed in all media under all conditions tested. An additional point for differentiation is that mannose inhibits the species more than it does the variety. The comparative growth rates are 9.2 for C. sorokiniana, 7.0 for C. sorokiniana var. atlanticensis and 5.0 for C. sorokiniana var. pacificensis.

Chlorella autotrophica var. atypica var. nov.

Figure (15)

Cells spherical to slightly ovoid, 4-9μ in diameter. Chromatophore cup-shaped filling less than 1/2 of the cell; on glucose in the light the chromatophore is shallow cup-shaped and granular with a cell diameter nearer to 12μ. Pyrenoid present.

Slow growth on inorganic media in the light; not supported by any sugars in darkness, no acceleration in the light. Acetate supports weak growth in darkness.

Casein hydrolysate, NH_3 and nitrate serve equally well as N sources.

Type culture number MCC 10 isolated by R. Lewin from sea-water.

Displaying a strong autotrophic tendency, the isolate can grow slowly on acetate. This growth was not as erratic as the growth of the species on acetate. In contrast to the species, the variety atypica has a clear pyrenoid. A preference for NH_3 as a N source could not be established for the variety. Morphologically the cells of the variety are a little larger than those of the species, also the chromatophore of the species is more cup-shaped, than that of the variety.

Chlorella anitrata spec. nov.

Figures (19,20,21)

Cells spherical, 5-10μ in diameter, nearer to 12μ on glucose in darkness. Chromatophore mantle-shaped, granular on glucose in darkness, always green. Pyrenoid present.

Grows well on inorganic media in the light. Glucose supports growth in darkness and lightly stimulates in light; mannose supports weak growth in the light and darkness; other sugars ineffective. Acetate supports growth in light and darkness.

NH_3 serves well and casein hydrolysate almost equally well as a N source; nitrate does not support growth when used as a N source. Cells grown on NH_4NO_3 may reach a diameter of 30-50μ without releasing any daughter cells. The color of the giant cells is green, in both inorganic and glucose media, and the chromatophore is mostly alveolar in appearance. The latter may be due to a high number of daughter cells within the mother cells, since in some isolated instances cup-shaped granular chromatophores filling only approximately 1/3 of the cells have been observed.

Yeast extract ineffective in inorganic and glucose media.

Type culture number MCC 12 isolated by Gross from Canal between Pompano and West Hollywood, Florida.

Except for Chlorella anitrata var. minor none of the other marine isolates showed a similar physiological pattern. Search in the monograph by Shihira and Krauss did not bring forth any species of the genus Chlorella with similar physiological characteristics. Those species of the genus which show little or no growth on nitrate do not show

clearcut nitrate inhibition. The species produces giant cells when grown on nitrate. Cytokinesis appears to take place in many of the giant cells but the daughter cells are never liberated to pursue independent growth.

Chlorella anitrata var. minor var. nov.

Figures (22,23,24)

Cells spherical, 3-6μ in diameter closer to 10μ when grown on glucose in darkness. Chromatophore cup-shaped, becoming very granular on glucose, always deep green. Pyrenoid present.

Grows well on inorganic media in light. Glucose supports growth in darkness and lightly accelerates in light; mannose supports weak growth in light and darkness. Other sugars are ineffective. Acetate supports growth in light and darkness.

NH_3 and casein hydrolysate serve equally well as N sources; nitrate does not support growth. Cells grown on NH_4NO_3 attain up to 30μ in diameter but no daughter cells are discharged. The color of the giant cells is deep green and the chromatophore alveolar.

Type culture number MCC 13 isolated by Gross from Intracoastal Waterway, Dania, Florida.

The physiological pattern of this isolate is the same as that of Chlorella anitrata. There is a difference in that there is a smaller size of the abnormal cells on nitrate.

The normally grown cells are also smaller than those of the species. The differences in the species and variety are minimal for the establishment of identity, however, the isolates are clearly different in this size range.

Chlorella glucotropha spec. nov.

Figures (25,26)

Cells ovoid, 2-5μ in diameter. Chromatophore cup-shaped, deep green in inorganic media changing to light green on glucose media. Pyrenoid visible.

Growth on inorganic media is slow. Glucose supports weak growth in the dark; other sugars are ineffective; mannose strongly inhibits growth in the light. Acetate supports weak growth in darkness; 1% acetate completely inhibits this organism.

Grows on nitrate or casein hydrolysate as a N source, but there is a preference for ammonium.

Yeast extract is ineffective in inorganic or glucose media.

Type culture number MCC 14 isolated by Gross from Salt Peter Creek, southeast of Chase, Maryland.

The species *Chlorella glucotropha* is distinct from the other species showing autotrophic characteristics in that it will grow in the dark on glucose and acetate--although poorly. It will grow well on all three nitrogen sources like *Chlorella verautotrophica* but unlike *C. verautotrophica* var. *miniata* which barely grows on NO_3.

Chlorella glucotropha var. *caribbeanensis* var. nov.

Figures (27,28)

Cells ovoid, 4-6μ in diameter. Chromatophore parietal, filling the cell, deep green changing to very pale green on glucose in darkness. Pyrenoid present.

Good growth on inorganic media. Glucose gives slight acceleration to light growth, and supports weak growth in darkness; no other sugar

effective in light or darkness. Acetate ineffective in light or darkness; 1% acetate reduced growth in the light.

Ammonium, nitrate, and casein hydrolysate serve equally well as N sources; in NH_3 and nitrate media, the cells have a tendency to stick to the walls of the test tubes.

Yeast extract does not accelerate growth.

Type culture number MCC 15 isolated by Gross from San Juan harbour, Puerto Rico.

C. glucotropha var. caribbeanensis shows the autotrophic tendency of the species. Both the species and the variety are ovoid and display a distinct pyrenoid.

C. glucotropha var. caribbeanensis differs from the species by a slightly higher growth rate in the light, for both inorganic and glucose medium, by a greater loss of chlorophyll in glucose in darkness and by a tendency of the cells to stick to the walls of test tubes under certain conditions. Also the species is strongly inhibited by mannose, whereas the variety is not; in 1% acetate the species is completely inhibited, the variety only moderately. Acetate supports slight growth in darkness for the species but not for the variety.

Chlorella verautotrophica spec. nov.

Figure (29)

Cells spherical to ovoid, 3-7μ in diameter. Chromatophore mantle-shaped. Pyrenoid present. Daughter cells tend to adhere after release from the mother cell.

Growth on inorganic media is slow. All sugars are ineffective

in light and darkness. Acetate is ineffective, 1% acetate completely inhibits growth in the light.

NH_3 serves well as a N source. Nitrate and casein hydrolysate are less effective but fair growth is supported.

Yeast extract is ineffective in any medium.

Type culture number MCC 16 a isolated by Gross from Pompano Beach, Florida. Identical strain, MCC 16 b, isolated from east of Annapolis, Chesapeake Bay, Maryland.

In inorganic medium, Chlorella autotrophica and Chlorella verautotrophica have the same dimensions and overall appearance, however sometimes the daughter cells of Chlorella verautotrophica tend to adhere after release.

Chlorella verautotrophica is a true autotroph.

Chlorella verautotrophica var. minuta var. nov.

Figure (30)

Cells spherical, 2-4μ in diameter. Chromatophore cup-shaped, or parietal filling the cell. No visible pyrenoid.

Slow growth on inorganic liquid media. Glucose and all other sugars ineffective in light and darkness; acetate ineffective in light and darkness.

NH_3 serves well as a N source; casein hydrolysate supports some growth; nitrate supports no growth.

There is no detectable stimulation by yeast extract.

Type culture number MCC 17 isolated by Gross from Canal Pompano to West Hollywood, Florida.

The variety exhibits the strong autotrophic characteristics of the

species. The cell size is reduced about one-half that of the species.

Chlorella pyrenoidosa Chick 1903:458

Figures (31,32)

Cells spherical to ovoid, 3-4μ in diameter, nearer to 10μ for some mother cells. Chromatophore cup-shaped in young cells, becomes mantle-shaped in older cells, deep green in inorganic media, almost colorless and granular on glucose in darkness. A conspicuous dark granule is often observed in each cell; developing daughter cells have one; older cells may have several. Pyrenoid present.

Grows well on inorganic media in the light. Glucose stimulatory in light, supports only weak growth in darkness. Galactose and mannose support weak growth in the light and darkness; fructose is ineffective. Acetate is stimulatory in light and supports very weak growth in darkness.

Ammonium and casein hydrolysate serve equally well as N sources; nitrate supports little, and sometimes no, growth at all; NH_4NO_3 serves as a N source without any ill effects due to the presence of nitrate.

Yeast extract may be slightly stimulatory in inorganic and glucose media.

Type culture number MCC 18 a isolated by Gross from 2 miles off Ocean City, Maryland (Atlantic). Identical strain MCC 18 b isolated from Canal Pompano to West Hollywood, Florida.

This is the first isolate obtained in this laboratory that in morphology and physiology matches that described by Chick in 1903.

Many times isolates of Chlorella have erroneously been ascribed to this species and the physiological literature is replete with references to Chlorella pyrenoidosa which are not tenable.

The outstanding characteristic is the clear preference for ammonia or compounds bearing the NH_2 group. Chick implies, but does not unequivocally state, that NO_3 does not serve as a N source. She also makes a strong point of the stimulation by glucose in the light, and the disappearance of chlorophyll in the dark. Another species which shows a preference for NH_3 is Chlorella variabilis Shihira and Krauss. However, this species is inhibited rather than stimulated by glucose in the light. Chlorella anitrata demonstrates the NH_3 preference but does not bleach in the dark and forms conspicuous giant cells on NO_3.

Chlorella parva spec. nov.

Figures (33,34)

Cells always spherical, 1-3μ in diameter, nearer to 5μ when grown on glucose media. Chromatophore smooth and cup-shaped, deep green; becomes granular and very pale green with a flat bowl-like appearance on glucose in darkness. Pyrenoid present.

Grows well on inorganic media in light; glucose very stimulatory in light, supports less growth in the dark than inorganic media in the light. Galactose supports excellent growth in the light, but only a trace in darkness; fructose mostly ineffective; mannose is moderately inhibitory to light growth, and does not support any growth in darkness. Acetate supports some growth in darkness, ineffective in the light; 1% acetate has an inhibitory effect on light growth equivalent

to mannose.

NH_3 and casein hydrolysate serve equally well as N sources; nitrate may be slightly preferred.

Yeast extract ineffective in inorganic or glucose media.

Type culture number MCC 19a isolated by Gross from Delaware Bay (near mouth), Delaware. Identical strain MCC 19b isolated from Delaware Bay (center), Delaware.

The metabolic pattern for this isolate does not readily compare with any known species of the genus. There are some resemblances to C. simplex, but not enough points of similarity exist to call them related. Chlorella parva is the smallest isolate in the collection. This species also had the most rapid rate of growth of any marine species on the shaker. It must be assumed that it is a new species.

Chlorella gravata spec. nov.

Figure (35)

Cells spherical, 2-5μ in diameter. Chromatophore shallow, bowl-shaped, sometimes net-like. Pyrenoid present. Liquid cultures tend to show many atypical cells.

Slow growth on inorganic agar media enriched with yeast extract.

The following list of media support very slow growth in the light only:

Inorganic medium, NH_4NO_3 as N source, and 0.01% yeast extract

Inorganic medium and 0.01% yeast extract and 0.1% glucose

Inorganic medium containing 50% natural sea-water

Full strength sea-water enriched with inorganic nutrients.

Growth factor requirements have not been clearly established since tests using thiamin proved inconclusive, but growth on media without yeast extract could not be achieved satisfactorily.

Type culture number MCC 20a isolated by Gross from Chesapeake Bay, off Rock Hall Landing, Maryland. Identical strain MCC 20b isolated from Chesapeake Bay, near Annapolis, Maryland.

The most outstanding feature of this species is its very slow growth on any media. All indications are that it has a growth factor requirement which is contained in yeast extract or natural sea-water. It failed to grow on any of the eleven vitamins supplied, nor were any amino acids or sugars capable of supporting growth. It is a difficult organism to study because of the very slow rate of growth. However it is potentially very interesting in that it may have an unusual growth requirement. It should be placed in the subgenus Auxenochlorella pending final resolution of its essential growth factor. It is significant that two isolates of this organism were obtained so it may be assumed not to be rare.

Chlorella salina Butcher 1952:179

Figures (36,37)

Cells spherical to slightly ovoid, 3-8μ in diameter, nearer to 8μ when grown on glucose. Chromatophore smooth and cup-shaped in young cells, slightly granular and mantle-shaped in older cells; deep green in inorganic media, pale green and coarsely granular on glucose in darkness. Pyrenoid present.

Slow growth on inorganic media. Glucose accelerates growth in

the light and supports very weak growth in darkness. Galactose stimulates growth in the light at least as much as glucose and supports weak growth in darkness; fructose ineffective in the light and darkness; mannose strongly inhibitory. Acetate weakly stimulatory in light and darkness; 1% acetate causes some inhibition of growth in the light.

Nitrate and casein hydrolysate serve equally well as N sources; growth on NH_3 as a N source is much reduced.

Yeast extract ineffective in the light and darkness.

Type culture number PL 86 isolated by Butcher.

Synonym: Chlorella ovalis Butcher

This isolate was presented as Chlorella salina nov. spec. by R. W. Butcher (3). Later in a personal communication he referred to it as C. salina Kufferath, which may be an older name that has preference, but no earlier reference to the name has been found.

The isolate CRA-1 treated as C. ovalis nov. spec. in Butcher's publication (3) supposedly is ovoid to spherical shaped, 2-4μ x 5-10μ in diameter, without a pyrenoid. He states this isolate differs from Chlorella sparkii Alvik in the division of mother cells into 8 rather than 2 daughter cells. Physiological and morphological examinations of CRA-1 sent to us by Dr. Butcher do not appear to be different from C. salina Butcher in any way. For this reason the two isolates should be considered as one and the same. It is proposed that Chlorella ovalis Butcher be dropped from the nomenclature, since it is the more recent of the names, as recommended in Article 63 of the

International Code of Botanical Nomenclature (10).

C. salina is quite unique in its inability to use NH_3 effectively. It is the only known isolate of Chlorella with this characteristic.

Chlorella sparkii Alvik 1934:31

Figure (38)

Cells ovoid, 4-5μ x 8-10μ in diameter. Chromatophore light green, cup-shaped in young cells, more lobed and parietal in older cells. Pyrenoid not present. Up to 16 daughter cells per mother cell.

Very slow growth in inorganic liquid and agar media. The cells characteristically attach to the walls of test tubes and cannot be shaken off. Due to the slow growth of this organism in all our media no tests of growth could be performed. Inoculations had to be repeated several times, in most cases, until some growth was established.

Addition of yeast extract was ineffective.

Neotype culture: LV 94 isolated by R. W. Butcher, maintained at the Marine Fisheries Laboratory.

Identical strain: PE 1 isolated by R. W. Butcher and described as Chlorella marina. Maintained at the Marine Fisheries Laboratory, Burnham-on-Crouch, Essex, England.

Synonym: Chlorella marina Butcher 1962:181

This organism was isolated from oyster tanks in Conway, N. Wales, and identified as being identical to C. sparkii Alvik. In a personal communication, Dr. Butcher indicated that this isolate differs from

C. stigmatophora Butcher (1940) by not having a pyrenoid. He stated that they are related since both reproduce by "simple division of a mother cell into two". However, we have observed up to 16 daughter cells in mother cells in the culture sent to us labeled as C. sparkii Alvik--as given in the amended description above.

Morphologically and physiologically, the characteristics of C. sparkii Alvik and C. marina Butcher appear identical. The description of C. marina Butcher as given by Dr. Butcher (3) seems to match the morphology of the isolate labeled by him as C. sparkii. It is significant that both these isolates came from oyster ponds--one in Scotland and the other in Norway.

Since C. sparkii Alvik as isolated by Dr. Butcher and C. marina Butcher are identical in our tests, it becomes necessary to reduce C. marina Butcher to synonomy.

Nomena inquirenda:

Chlorella stigmatophora Butcher 1952:180

The isolate sent to us under this name could not be purified on time to be used in this study. A morphological examination of the liquid transport culture as received has shown the following: the cells are ovoid, 3-5μ in diameter with a shallow cup-shaped chromatophore which does not fill the cell. There is a pyrenoid present. In addition, each cell displayed one to several dark granules. Presumably these were the dark brown bodies designated as being stigmata by Dr. Butcher.

Chlorella pyrenoidosa type *halophyla*

Nakamura 1963:40

This isolate has only been mentioned and is not adequately described. Furthermore it was only tentatively named by Nakamura and is therefore a *nomen provisiorum* and is not valid under the International Rules of Botanical Nomenclature. This isolate was not available for this study.

Physiological Key for the Marine Isolates of Chlorella

1. Organisms with no apparent growth factor requirements.

A. Organisms not growing well in the light on the N sources, NO_3 or NH_3.

1. Organism cannot use NO_3 as a N source, good growth on NH_3.

a. Presence of NO_3 is not inhibitory, during growth on some other N source.

b. Good growth on glucose in darkness; acetate strongly accelerates growth on inorganic medium in the light - C. pyrenoidosa.

bb. No growth on glucose in darkness; acetate does not accelerate growth in inorganic medium in the light - C. verautotrophica var. minuta.

aa. Presence of NO_3 is inhibitory during growth on some other N source.

b. Average cell diameter of 8μ in inorganic medium; 12μ in glucose medium in darkness - C. anitrata.

bb. Average cell diameter of 5μ in inorganic medium; 8μ in glucose medium in darkness - C. anitrata var. minor.

2. Organism using NO_3 as a N source, very weak growth on NH_3. Strongly inhibited by mannose - C. salina.

B. Organisms growing well in the light on the N sources, NO_3 or NH_3.

1. Organisms do not grow well on glucose in the light or in

darkness.

a. Not inhibited by mannose in the light.

b. Light growth on acetate in darkness - Chlorella autotrophica var. atypica.

bb. No growth on acetate in darkness.

c. No growth on glucose in light or darkness - C. verautotrophica.

cc. Always slight growth on glucose in the light and darkness - C. glucotropha var. caribbeanensis.

aa. Strongly inhibited by mannose in the light. Good growth on acetate in darkness - C. glucotropha.

2. Organisms grow well on glucose in the light or in darkness.

a. Glucose promotes little or no acceleration of growth in the light.

b. Mannose inhibits growth in the light.

c. Good growth on fructose in light and darkness, slight growth on lactose in darkness - C. regularis var. umbricata.

cc. No growth on fructose in light and darkness, no growth on lactose - C. nocturna.

bb. Mannose does not inhibit growth in the light. Good growth on mannose in darkness - C. acuminata var. pompanoensis.

aa. Glucose promotes good growth in the light (O.D. at least

0.3 units over growth on inorganic media in the light).

b. No other sugars besides glucose promote growth.

c. Strongly inhibited by mannose in the light, no growth in the dark - C. infusionum var. acetophila.

cc. Mannose promotes light growth in the light and darkness - C. pyrenoidosa.

bb. Other sugars besides glucose promote good growth.

c. Good growth on mannose in the light and darkness.

d. Fructose promotes good growth in the light only. Galactose also promotes good growth in the light - C. emersonii var. globosa.

dd. Fructose promotes good growth in the light and darkness. Galactose ineffective in the light - C. regularis var. miniata.

cc. No growth on mannose in the light and darkness.

d. Acetate does not accelerate growth in the light. Excellent growth on glucose and galactose in the light - C. parva.

dd. Acetate accelerates growth in the light by at least 100%.

e. No growth at 38°C.

f. Little or no growth on NH_3 as a N source - C. salina.

ee. Excellent growth at 38°C.

f. No clumping of cells; average cell diameter 4μ - C. sorokiniana var. atlanticensis.

ff. Cells always clump; average cell diameter 4μ - C. sorokiniana var. pacificensis.

2. Organism with apparent growth factor requirement. Addition of 0.1% yeast extract promotes growth - C. gravata.

Figure 1,2 Top: Chlorella nocturna Shihira and Krauss MCC #3. Photographed from actively growing liquid culture. X900. Left: Cultured on inorganic medium. Right: Cultured on glucose medium in darkness.

Figure 3,4 Middle: Chlorella regularis (Artari) Oltmanns var. umbricata Shihira and Krauss MCC #4. Photographed from actively growing liquid culture. X900. Left: Cultured on inorganic medium. Right: Cultured on glucose medium in darkness.

Figures 5,6 Bottom: Chlorella regularis var. minima var. nov. MCC #5. Photographed from actively growing liquid culture. X900. Left: Cultured on inorganic medium. Right: Cultured on glucose medium in darkness.

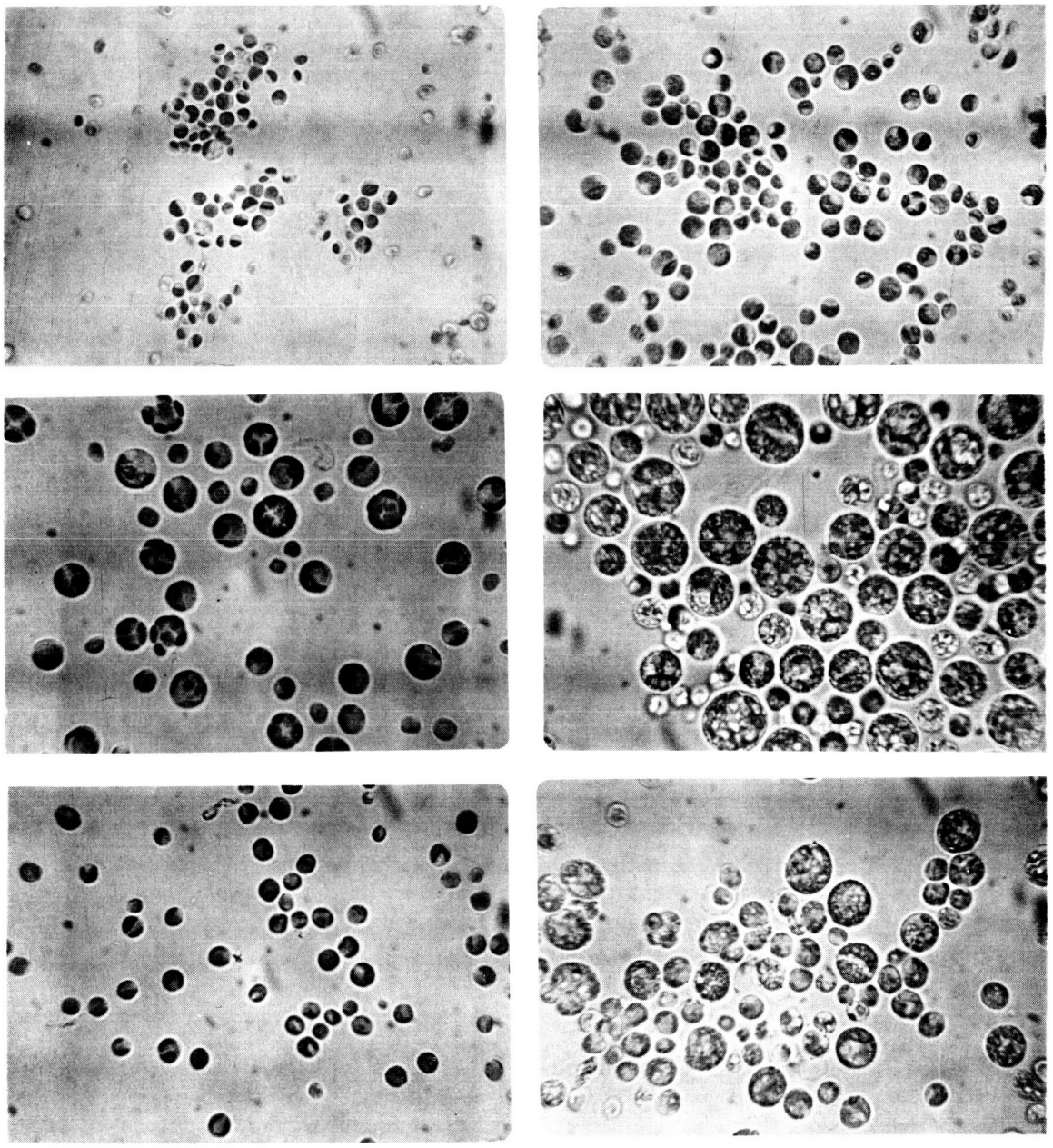


Figure 7,8 Top: Chlorella infusionum var. acetophila var. nov. MCC #6. Photographed from actively growing liquid culture. X900. Left: Cultured on inorganic medium. Right: Cultured on glucose medium in darkness.

Figure 9,10 Middle: Chlorella acuminata var. pompanoensis var. nov. MCC #7. Photographed from actively growing liquid culture. X900. Left: Cultured on inorganic medium. Right: Cultured on glucose medium in darkness.

Figure 11,12 Bottom: Chlorella sorokiniana var. atlanticensis var. nov. MCC #8. Photographed from actively growing liquid culture. X900. Left: Cultured on inorganic medium. Right: Cultured on glucose medium in darkness.

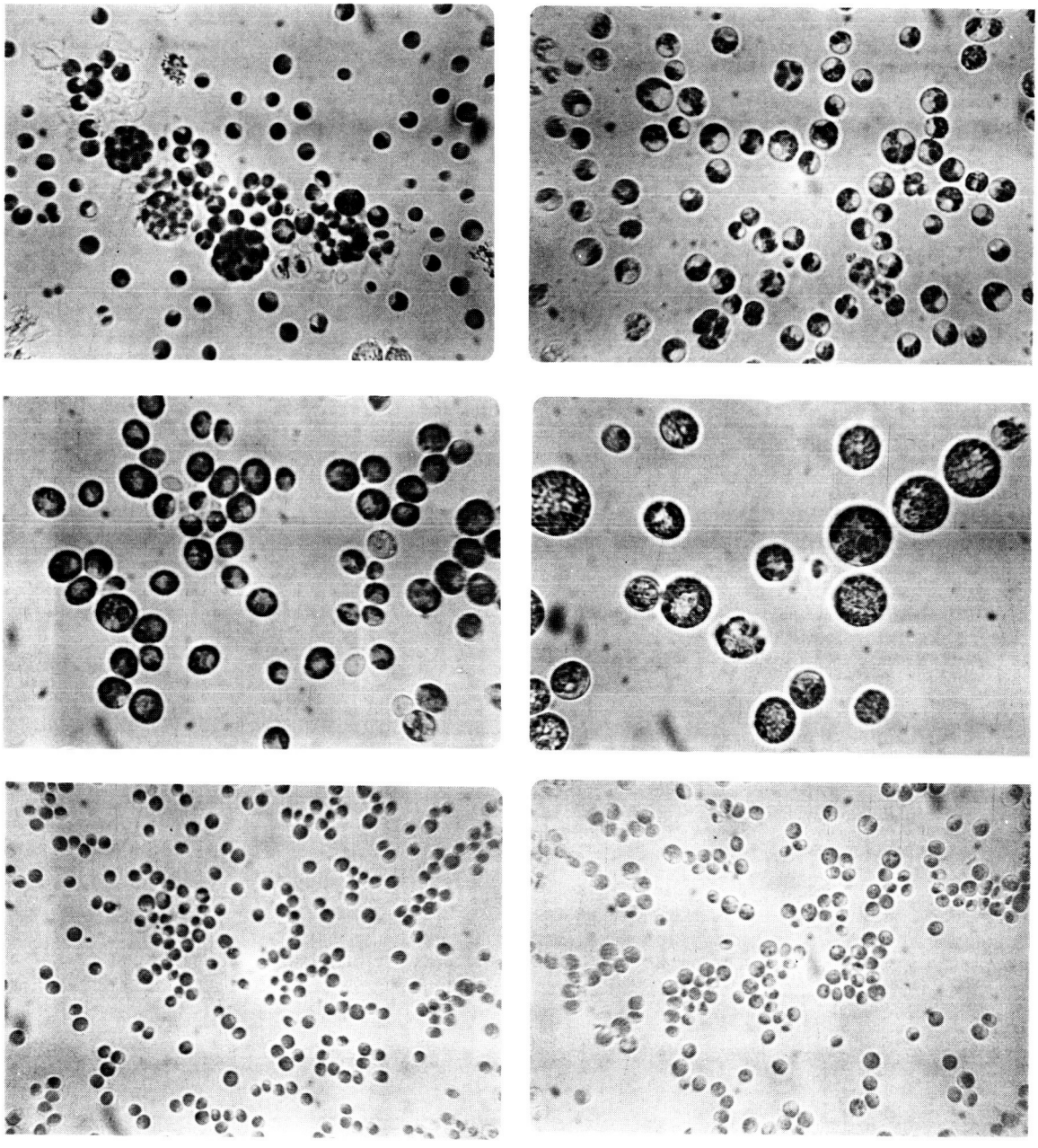
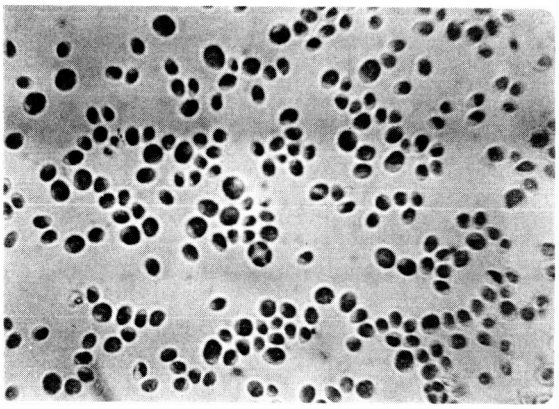
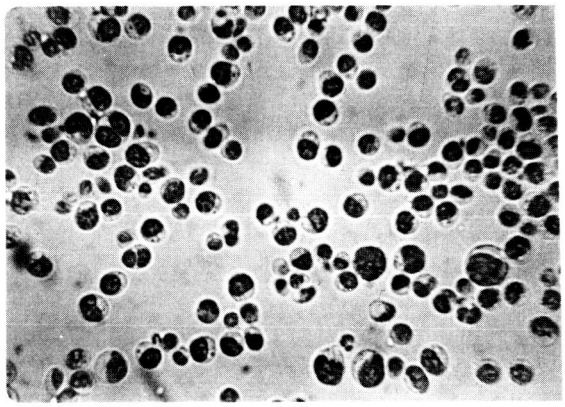


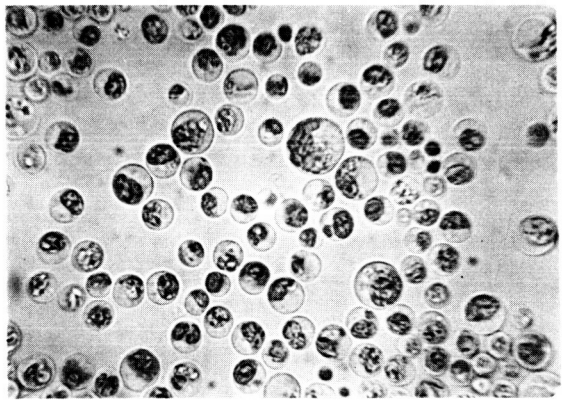
Figure 13,14 Top: Chlorella sorokiniana var. pacificensis var. nov. MCC #9. Photographed from actively growing liquid culture. X900. Left: Cultured on inorganic medium. Right: Cultured on glucose medium in darkness.

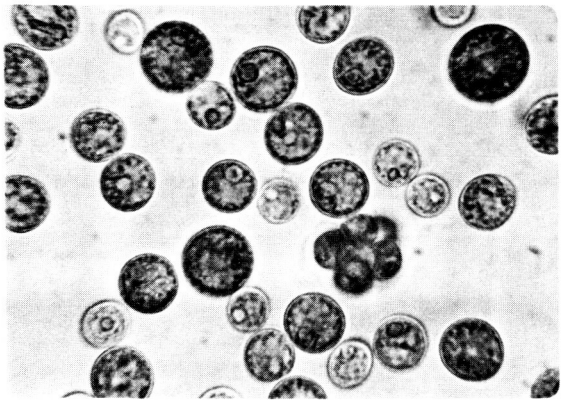
Figure 15 Middle: Chlorella autotrophica var. atypica var. nov. MCC #10. Photographed from actively growing liquid culture. X900. Cultured on inorganic medium.

Figure 16,17 Bottom: Chlorella emersonii var. globosa Shihira and Krauss. MCC #11. Photographed from actively growing liquid culture. X900. Left: Cultured on inorganic medium containing NO_3 as a N source. Right: Cultured on glucose medium in darkness.









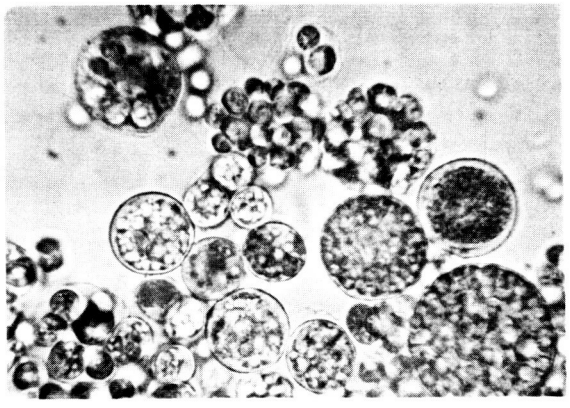
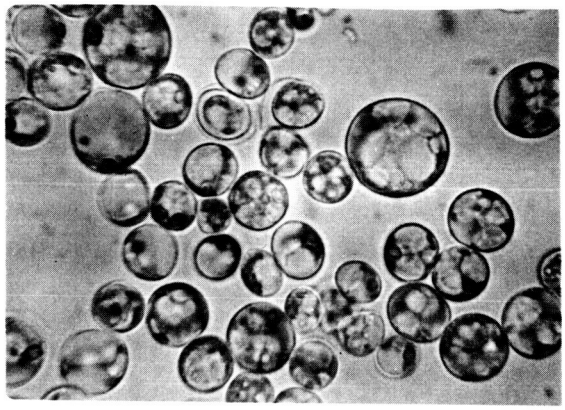
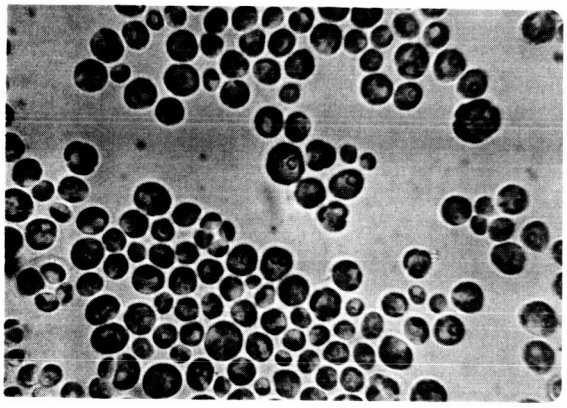


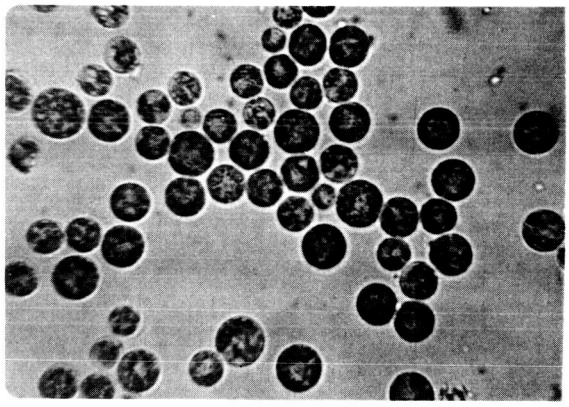
Figure 18 Top: Chlorella emersonii var. globosa Shihira and Krauss. MCC #11. Photographed from actively growing liquid culture. X900. Cultured on inorganic medium containing NH_3 as a N source.

Figure 19,20 Middle: Chlorella anitrata spec. nov. MCC #12. Photographed from actively growing liquid culture. X900. Left: Cultured on inorganic medium containing NH_3 as a N source. Right: Cultured on glucose medium in darkness.

Figure 21 Bottom: Chlorella anitrata spec. nov. MCC #12. Photographed from actively growing liquid culture. X900. Cultured on inorganic medium containing NO_3 as a N source.







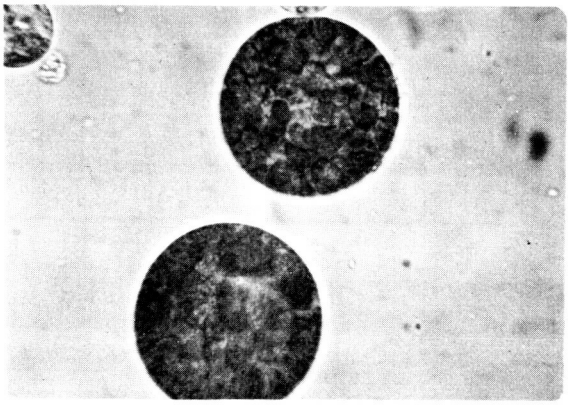
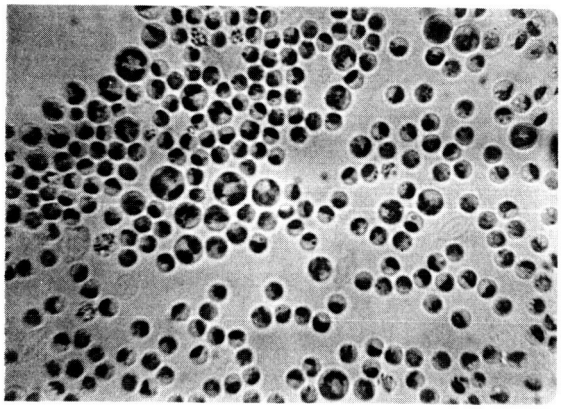
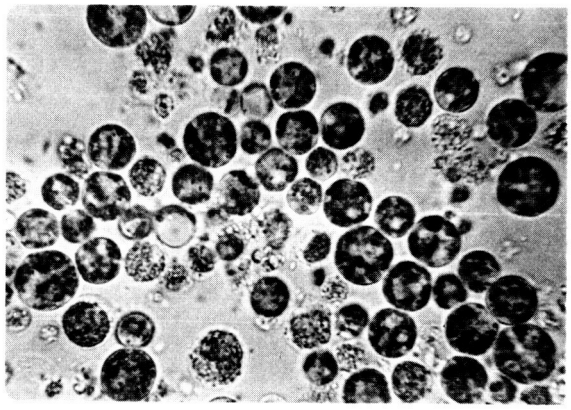


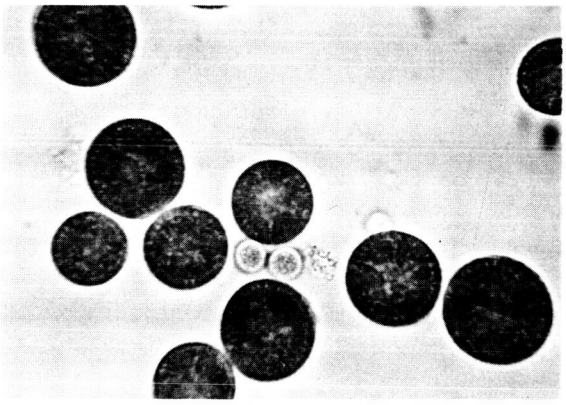
Figure 22,23 Top: Chlorella anitrata var. minor var. nov. MCC #13. Photographed from actively growing liquid culture. X900. Left: Cultured on inorganic medium containing NH_3 as a N source. Right: Cultured on glucose medium in darkness.

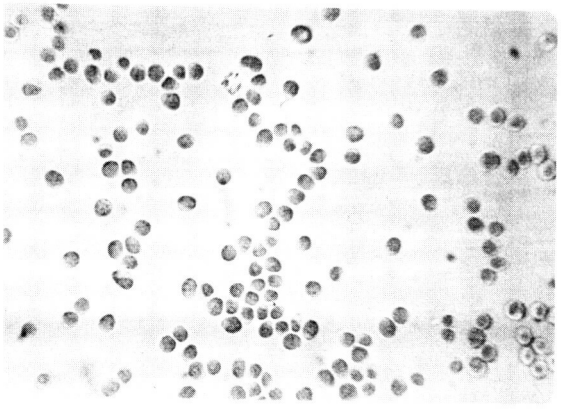
Figure 24 Middle: Chlorella anitrata var. minor var. nov. MCC #13. Photographed from actively growing liquid culture. X900. Cultured on inorganic medium containing NO_3 as a N source.

Figure 25,26 Bottom: Chlorella glucotropha spec. nov. MCC #14. Photographed from actively growing liquid culture. X900. Left: Cultured on inorganic medium. Right: Cultured on glucose medium in darkness.









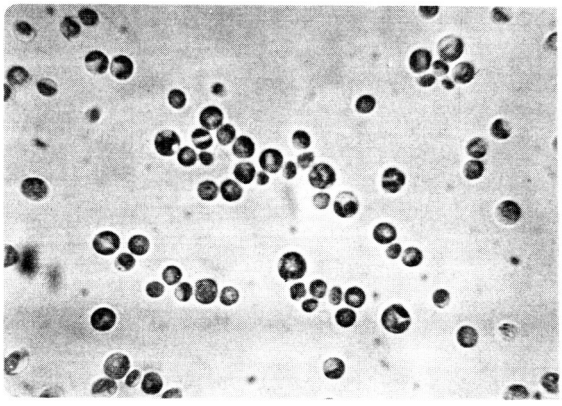
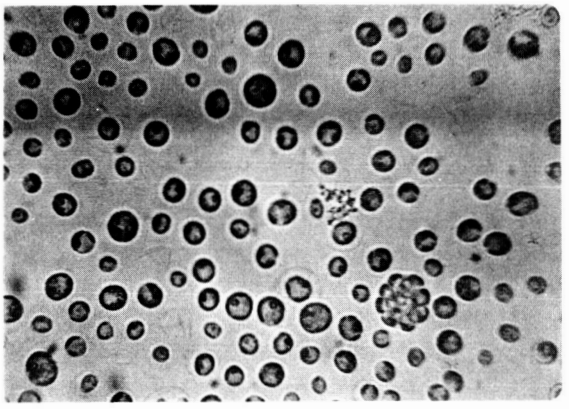
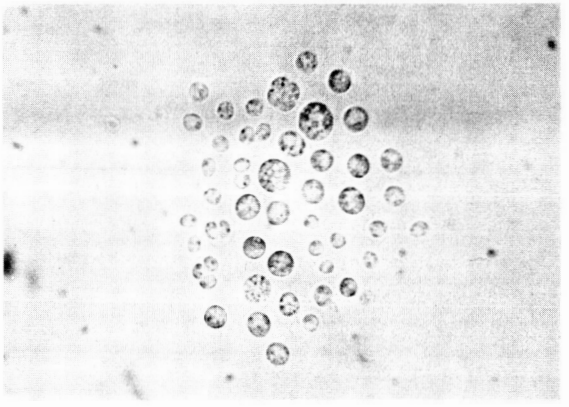


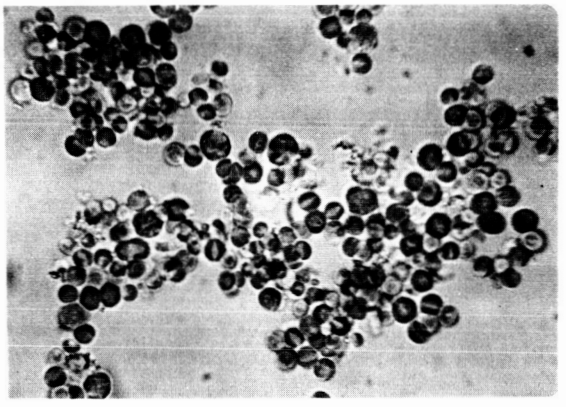
Figure 27,28 Top: Chlorella glucotropha var. caribbeanensis var. nov. MCC #15. Photographed from actively growing liquid culture. X900. Left: Cultured on inorganic medium. Right: Cultured on glucose medium in darkness.

Figure 29 Middle: Chlorella verautotrophica spec. nov. MCC #16. Photographed from actively growing liquid culture. X900. Cultured on inorganic medium.

Figure 30. Bottom: Chlorella verautotrophica var. minuta var. nov. MCC #17. Photographed from actively growing liquid culture. X900. Cultured on inorganic medium.







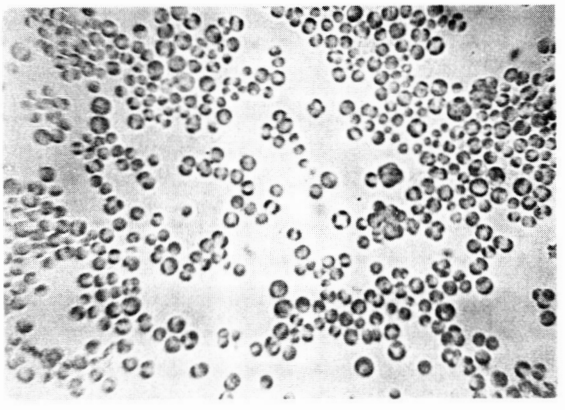


Figure 31,32 Top: Chlorella pyrenoidosa Chick MCC #18. Photographed from actively growing liquid culture. X900. Left: Cultured on inorganic medium. Right: Cultured on glucose medium in darkness.

Figure 33,34 Middle: Chlorella parva spec. nov. MCC #19. Photographed from actively growing liquid culture. X900. Left: Cultured on inorganic medium. Right: Cultured on glucose medium in darkness.

Figure 35 Bottom: Chlorella gravata spec. nov. MCC #20. Photographed from actively growing liquid culture. X900. Cultured on inorganic medium.

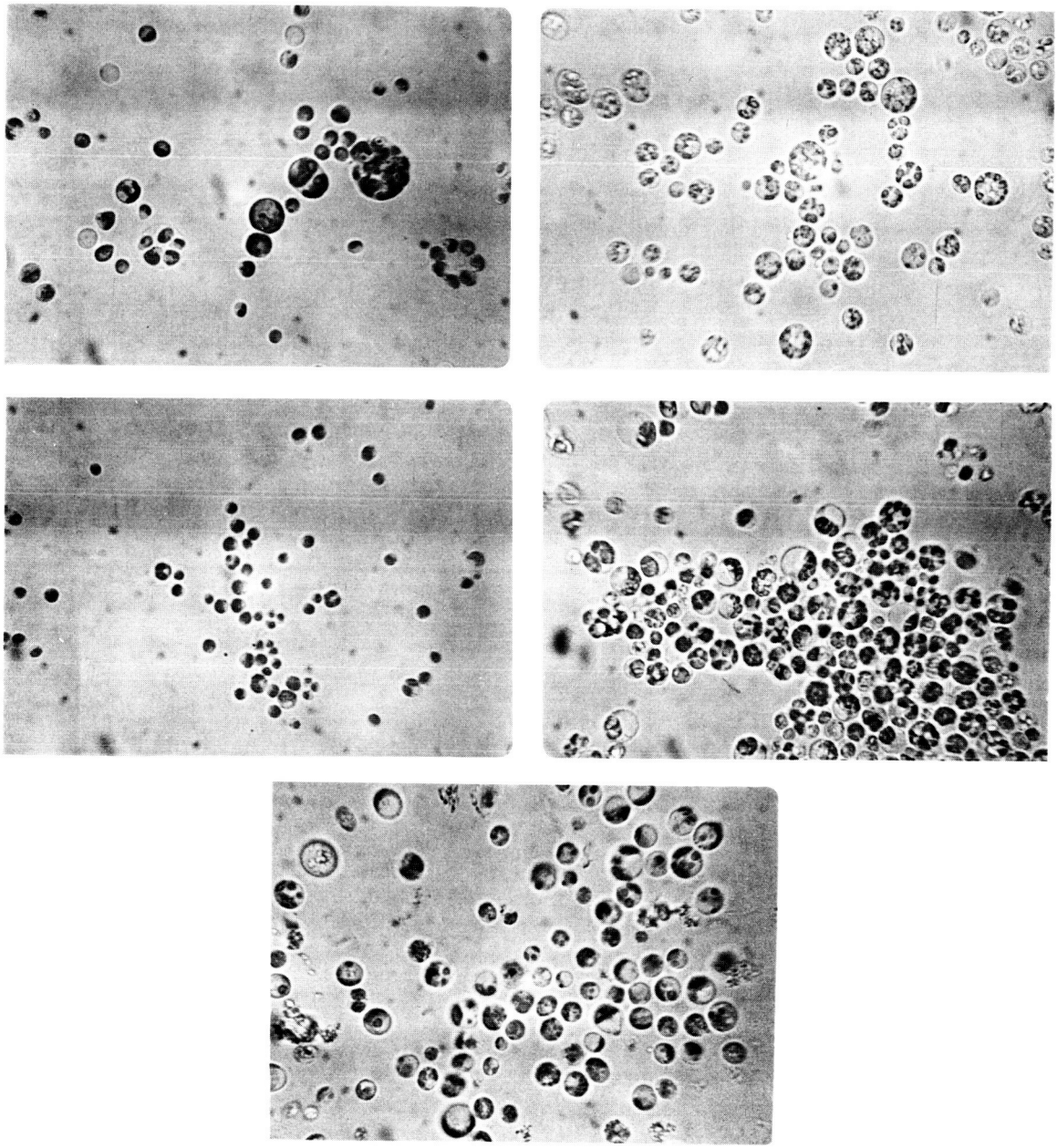
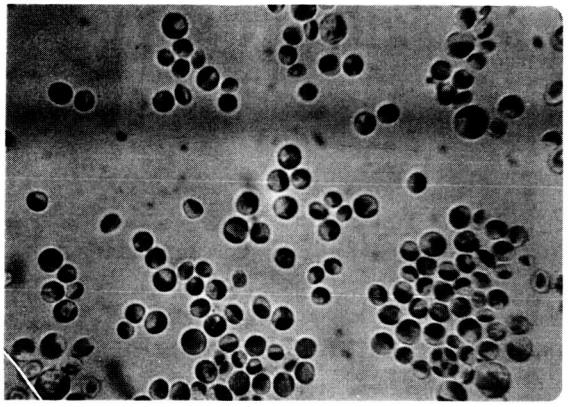
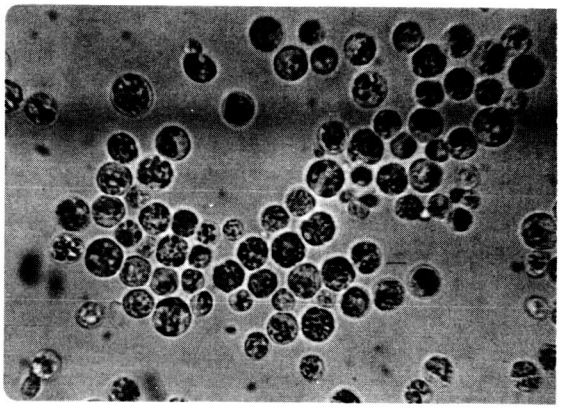
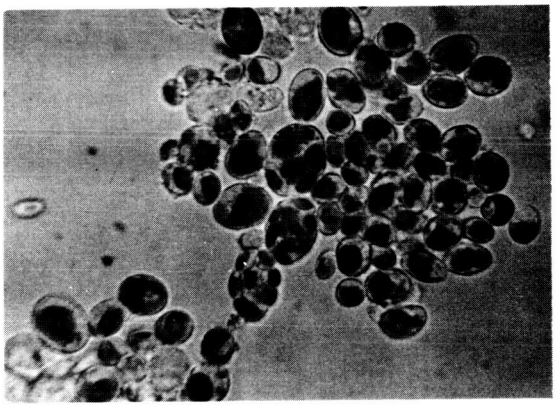


Figure 36,37 Top: Chlorella salina Butcher PL 86. Photographed from actively growing liquid culture. X900. Left: Cultured on inorganic medium. Right: Cultured on glucose medium in darkness.

Figure 38 Bottom: Chlorella sparkii Alvik CV 94. Photographed from actively growing liquid culture. X900. Cultured on inorganic medium.







N65-15803

15805

Summary

One hundred samples of sea water and brackish water were collected from widely separated areas of the Atlantic and Pacific coasts and examined for species of the genus Chlorella. Twenty-nine unialgal isolates were freed from contaminants by standard bacteriological techniques. Included in this study were four previously isolated salt-water species from Europe.

A series of physiological tests were applied in order to determine relationships between the different isolates. The tests included growth on selected sugars in the light and darkness; determination of the ability to use NO_3, NH_3 or casein hydrolysate as nitrogen sources; the toxicity or suitability of acetate as a carbon source; and the identification of vitamin requirements. The effects of enriched natural sea water on growth rates were studied employing a group of fresh-water species for comparison.

Morphological characteristics of the marine isolates when grown autotrophically in the light and heterotrophically in darkness on glucose were examined. Changes in cell morphology due to different nitrogen sources were indicated. Cells from standardized cultures were photographed in color.

Eleven isolates were eliminated as duplicates. Three isolates were identified as being identical to previously known fresh-water species. Seven isolates were established as new varieties of known species of the genus. Eight isolates were found to be new species or

varieties of new species of Chlorella. The physiological and morphological relationships between the marine species were discussed, and where applicable, comparisons have been made with previously known species of the genus.

APPENDIX

The procedures for the isolation of marine species of Chlorella lead to the isolation and purification of a group of green algae that can be called close relatives of Chlorella. Such organisms were unicellular, reproduced by the formation of aplanospores, and had the same types of chromatophores encountered in Chlorella. They differ from Chlorella by minor morphological characteristics. In most cases, it was possible to suggest a genus to which the isolates belong, but no effort was made to assign specific names. Brief descriptions of certain of these isolates will serve to acquaint the reader with the nature of allied genera encountered in sea water.

Isolate B-1

Collection: June 21, 1962, at ocean shore north of Rehoboth, Maryland.
Salinity: 36000 ppm.

Normally spherical cells; diameter 2-5μ; mantle-shaped chromatophore; pyrenoid indistinct. The organism when grown on inorganic medium and yeast extract exhibits normal spherical Chlorella-like cells, the mother cells of which contain 4-8 daughter cells. Many atypical cells can be observed. Cells are elongated up to 20μ, some of them thin (2-3μ) and others fairly thick (6-8μ); some of these long cells are bent to form a complete circle and the ends almost touch; other cells are ovoid or kidney-shaped; one cell was cigar-shaped with a spherical structure at one end. The chromatophore in

elongated cells is band-shaped and parietal. The great variety of shapes seems to exclude the possibility of a mixed culture. The organism may not have been grown in a suitable medium, or it may suffer from a genetic disorder which induces the production of aberrant forms.

Of all the liquid media on which this organism was tested, only 50% natural sea water inorganic medium, and medium containing 0.01% yeast extract and NH_3 for a nitrogen source supported growth. The rate of growth in both cases was extremely slow. No genus has been found which matches the structure of this isolate.

Isolate B-2

Collection: July 5, 1962, at Bay View Shore, Chincoteague Bay, Maryland

Salinity: above 20,000 ppm

Ovoid cells; averaging 6μ long and 3-4μ wide; pale green cup-shaped chromatophore becomes mantle-shaped in older cells; distinct pyrenoids present; mother cells with 4-16 daughter cells; the isolate is typified by many atypical cells in all media. The organism grows very slowly on basal medium. Glucose, galactose, and mannose support weak growth in the dark; a slight stimulation may occur on glucose and mannose in the light. Very thin growth on agar slants. No growth on casein hydrolysate, slight growth on NH_3 and growth equivalent to basal medium in nitrate marks the nitrate pattern of the organism. The organism is strongly inhibited by 1% acetate. Yeast extract is ineffective. The chlorophyll is easily lost from the cells of this isolate.

The organism is hard to place into any taxonomic scheme due to many different shapes of the cells. However Stichococcus (Ulothricales) produces similar looking cells by fission which could not be observed in this isolate.

Isolate B-3

Collection: July 9, 1962, in Canal Pompano, Florida.

Salinity: high (tidal waters).

July 9, 1962, off Aberdeen, Chesapeake Bay, Maryland.

Salinity: approximately 8000 ppm.

Cells ovoid; diameter 4-10μ, cup-shaped smooth chromatophore; always 4 daughter cells.

The organism is stimulated over growth in basal medium by glucose, galactose, and acetate in both light and dark. 1.0% acetate is strongly inhibitory.

Both isolates display an identical physiological and morphological pattern and are considered the same species. The organism belongs to the genus Westella because daughter cells remain bound together by filaments.

Isolate B-4

Collection: July 6, 1962, off Lewis, Delaware Bay, Delaware.

Salinity: near 36,000 ppm.

Ovoid cells; diameter 3-6μ; mantle-shaped chromatophore; pyrenoid present; always 4 daughter cells. After release from the mother cells due to a breakdown of the cell wall the daughter cells stay attached to each other by thin thread-like structures. As soon as the cells grow bigger in size, the appendages are lost. These threads could be pieces of the old mother cell wall.

Sugars are ineffective in light and dark. Acetate supports growth in light and dark, but the organism is completely inhibited by 1.0% acetate. This organism belongs to the genus Westella.

Isolate B-5

Collection: July 10, 1962, at Pompano Beach, Florida.

Salinity: approximately 36,000 ppm.

Spherical cells; diameter 4-10μ; chromatophore cup-shaped in very young cells, becomes alveolar in larger cells; deep green in basal medium, light green in glucose and dark; distinct pyrenoid present; 8-16 daughter cells. Each cell has one or more dark granules in basal medium. The cell wall is somewhat thicker and each cell has at least one unipolar swelling or round nodule which looks like a thickening of the wall. Cells having two nodules do not have them directly opposite.

Glucose supports some growth in light and dark; no other organic source is effective with or without yeast extract. Growth is reduced in 1% acetate.

Judging by the nodules, the organism probably should be placed in the genus Oocystis.

Isolate B-6

Collection: August 9, 1962, at Chincoteague Bay, Maryland.

Salinity: more than 20,000 ppm.

Spherical cells; diameter 3-7μ; cup-shaped chromatophore becoming mantle-shaped in older cells; pyrenoid present; 1-2 dark granules per cell; 4-32 daughter cells. A localized thickening in the cell wall gives the appearance of a polar nodule, similar to those

observed in Exentrosphaera, but the isolate differs in size and chromatophore from the latter organism. The organism is not stimulated by added carbohydrates in light or dark. It probably should be classed as Oocystis.

Isolate B-7

Collection: September 26, 1962, Harbor of Acapulco, Mexico.

Salinity: 36,000 ppm.

Ovoid cells; diameter 2-4μ x 3-6μ; mantle-shaped chromatophore; pyrenoid visible; orange stigma present; 4-8 daughter cells sometimes tend to stick in short chains.

At times the cells seem to have a tiny nodule on each pole. The organism is stimulated by glucose in light and dark; mannose and acetate accelerate growth much in the light and, like galactose, little in the dark.

This organism probably belongs to the genus Oocystis.

Isolate B-8

Collection: May 17, 1962, at Tall Timber Cove, St. Mary's County, Maryland.

Salinity: 12,000 ppm.

Round to oval cells; diameter 3-6μ; chromatophore cup-shaped in younger cells; pyrenoid present; normally 2-4 daughter cells.

There is an average of 3 stiff and very thin spines polarly attached to the cells.

None of the sugars tested supported growth in the dark, but some stimulated growth in the light (see appendix table 1).

It is suggested that this organism belongs to the genus Lagerheimia section Chodatella, a member of Oocystaceae.

Isolate B-9

Collection: July 10, 1962, at Intracoastal Waterway near Dania, Florida.

Salinity: near 36,000 ppm.

Round to slightly ovoid cells; diameter 2-5μ; chromatophore mantle-shaped; pyrenoid present; normally 4 daughter cells.

Thin, 3-4μ long spines are visible around the cells. The spines may originate from a basal granule.

Glucose stimulates growth over basal medium in both light and dark. This organism probably belongs to Lagerheimia section Eulagerheimia.

Isolate B-10

Collection: July 10, 1962, at Canal Pompano, Florida

Salinity: Tidal waters.

Ovoid cells; diameter 2-5μ; cup-shaped chromatophore; each cell shows one dark granule; 4 daughter cells.

Each cell had 3-5 spines which are very thin and may be missed even under oil immersion.

The isolate is not stimulated by any of the organic nutrients used.

It is suggested that this organism belongs to the genus Lagerheimia section Chodatella.

Isolate B-11

Collection: September 10, 1962, at ocean coast near San Juan, Puerto Rico.

Salinity: approximately 36,000 ppm.

Ovoid cells; diameter 2-5μ; granular chromatophore fills the cells; pyrenoid is distinct; 2-4 daughter cells.

Four to five long, straight spines approximately 5μ long originate from each pole. The organism is stimulated by glucose and acetate in light and dark.

It probably belongs to the genus Lagerheimia section Chodatella.

Isolate B-12

Collection: July 10, 1962, in Chesapeake Bay, Southeast of Solomons Island.

Salinity: approximately 15,000 ppm.

Ovoid cells; diameter 4-10μ; granular chromatophore fills most of the cells. Pyrenoid present; several dark granules per cell; 2-4 daughter cells. The cells are covered completely by short thin setae.

Growth is not supported by any of the carbohydrates tested.

The organism probably belongs to the genus Bohlinia.

Isolate B-13

Collection: July 9, 1962, at Chesapeake Bay, off Kent Point.

Salinity: approximately 15,000 ppm.

Slightly ovoid cells; diameter 1-3μ except when cells elongate up to 8μ long but do not get wider. Normally 4 daughter cells can be observed, but the elongated cells seem to divide by fission. Chromatophores are cup-shaped in regular cells and girdle-shaped in the elongated cells.

This isolate grew only in basal medium containing 50% natural salt water.

Another isolate from Chester River Basin, Chesapeake Bay, displayed

the same characteristics, and was considered to be the same organism. No tentative identification has been made.

Appendix Table 1 gives a relative comparison between autotrophic growth and heterotrophic growth that is supported by four sugars and acetate in light and darkness. None of the isolates showed any stimulation from the addition of sucrose or lactose during one week of culture. However Isolate B-3 showed some growth on sucrose in the dark when the tubes were standing for six weeks.

Appendix Table 1

Growth Measurements of Various Marine Isolates of Organisms Resembling *Chlorella*

After 5 days Incubation in Light and Darkness*

Isolate	Tentative Genus	Basal Medium		D-(d)-Glucose		D-(-)-Fructose		D-(+)-Galactose		D-(+)-Mannose		Sodium Acetate	
		L	D	L	D	L	D	L	D	L	D	L	D
B-1		0	0	0	0	0	0	0	0	0	0	0	0
B-2		1	0	2	1	1	0	1	1	2	1	1	0
B-3	*Westella*	2	0	5	4	2	0	4	1	2	0	5	3
B-4	*Westella*	2	0	2	0	2	0	2	0	2	0	3	1
B-5	*Oocystis*	2	0	1	0	2	0	2	0	2	0	2	0
B-6	*Oocystis*	2	0	2	0	2	0	2	0	2	0	2	0
B-7	*Oocystis*	2	0	5	2	2	0	2	1	4	1	4	1
B-8	*Lagerheimia*	3	0	5	0	4	0	4	0	4	0	4	0
B-9	*Lagerheimia*	4	0	5	2	4	0	4	0	4	0	4	1
B-10	*Lagerheimia*	3	0	3	1	3	0	3	0	3	0	3	0
B-11	*Lagerheimia*	4	0	5	1	4	0	4	0	4	0	5	1
B-12	*Bohlinia*	4	0	4	0	4	0	4	0	4	0	4	1
B-13		0	0	0	0	0	0	0	0	0	0	0	0

* 0=no growth; 1=O.D. more than .2; 2=O.D. .2-.4; 3=O.D. .4-.6; 4=O.D. .6-1; 5=O.D. more than 1.

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